UNIVERSIDADE DE LISBOA

FACULDADE DE FARMÁCIA

DECIPHERING THE INVOLVEMENT OF S100B-RAGE AXIS IN INFLAMMATION-ASSOCIATED MYELIN DAMAGE

Gisela Filipa Assunção Santos

Orientador(es): Prof. Doutora Adelaide Maria Afonso Fernandes Borralho
Prof. Doutora Dora Maria Tuna de Oliveira Brites

Tese especialmente elaborada para a obtenção do grau de **Doutor em Farmácia**, na especialidade de **Biologia Celular e Molecular**

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Júri

Presidente: Doutora Matilde da Luz dos Santos Duque da Fonseca e Castro, Professora Catedrática e Diretora da Faculdade de Farmácia da Universidade de Lisboa

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- Doutora Sara Alves Xapelli, Investigadora Auxiliar Faculdade de Medicina da Universidade de Lisboa;
- Doutora Adelaide Maria Afonso Fernandes Borralho, Professora Auxiliar Faculdade de Farmácia da Universidade de Lisboa, Orientadora

2018

This thesis was supervised by:

Professor Adelaide Fernandes, PhD (supervisor)

Assistant Professor

Research Institute for Medicines (iMed.ULisboa)

Faculty of Pharmacy, Universidade de Lisboa

Professor Dora Brites, PhD (co-supervisor)

Research Coordinator

Head of Neuron Glia Biology in Health and Disease

Research Institute for Medicines (iMed.ULisboa)

Faculty of Pharmacy, Universidade de Lisboa

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Neuron Glia Biology in Health and Disease Group, Research Institute for Medicines (iMed.ULisboa), Faculty of Pharmacy, Universidade de Lisboa

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3. Gomes C, **Santos G**, Falcão AS, Brites D. Early differentiating astroglial mouse progenitors share common protein signatures with GL261 glioma cells. J Stem Cell Regen Biol 2016 2(2): 77-91.

4. Torrado E, Gomes C, **Santos G**, Brites D, Falcão AS. Directing mouse embryonic neurosphere differentiation towards an enriched neuronal population. Int J Dev Neurosci 2014; 37:94-9.

Book Chapters

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Abbreviations

BBB	Blood-brain barrier
BSA	Bovine serum albumin
cAMP	2′,3′-cyclic adenosine monophosphate
CNP	2’,3’-cyclic nucleotide 3’-phosphodiesterase
CNS	Central nervous system
CNTF	Ciliary neurotrophic factor
COX	Cyclooxygenase
CP	Cerebral palsy
CSF	Cerebrospinal fluid
DIV	Days *in vitro*
DMEM	Dulbecco’s modified Eagle’s medium
DMF	Dimethyl fumarate
DMTs	Disease modifying therapies
DN-RAGE	Dominant-negative form of RAGE
E	Embryonic day
EAE	Experimental autoimmune encephalomyelitis
EGF	Epidermal growth factor
EM	Esclerose múltipla
EPO	Erythropoietin
FBS	Fetal bovine serum
FGF	Fibroblast growth factor
GFAP	Glial fibrillary acidic protein
GW	Gestational weeks
HBSS	Hank’s Balanced Salt Solution
HMGB1	High mobility group box 1
Iba1	Ionized calcium-binding adapter molecule 1
IFN	Interferon
IGF-1	Insulin-like growth factor 1
IL	Interleukin
iNOS	Inducible NO synthase
IVH	Intraventricular hemorrhage
LPA	Lysophosphatidic acid
LPC	Lysophosphatidylcholine
MAG	Microtubule associated protein
MBP	Myelin basic protein
MF	Microfilaments
MHC	Major histocompatibility complex
MHV	Mouse hepatitis virus
MOG	Myelin oligodendrocyte glycoprotein

MRF	Myelin regulatory factor
MS	Multiple Sclerosis
MT	Microtubules
N-WASP	Neuronal Wiskott-Aldrich syndrome protein
NF-κB	Nuclear factor-kappaB
NG2	Neural-glial antigen 2
NGF	Neural growth factor
nM	Nanomolar
NO	Nitric oxide
Nrf2	Nuclear factor erythroid 2-related factor 2
OCSC	Organotypic cerebellar slice cultures
OL	Oligodendrocytes
OPC	Oligodendrocyte precursor cells
P	Post-natal
PDGF	Platelet-derived growth factor
PDGFR	Platelet-derived growth factor receptor
PLP	Myelin proteolipid protein
PPMS	Primary progressive multiple sclerosis
PVL	Periventricular leukomalacia
RAGE	Receptor for advanced glycation end-products
ROCK	Rho-associated protein kinase
ROS	Reactive oxygen species
RRMS	Relapsing remitting multiple sclerosis
SC	Spinal cord
Sip-1	Smad-interacting protein-1
SPMS	Secondary progressive multiple sclerosis
sRAGE	Soluble RAGE isoform
TGF-β	Transforming growth factor β
TMEV	Theiler's murine encephalomyelitis virus
TNF-α	Tumor necrosis factor α
TNFR	Tumor necrosis factor receptor
WASP	Wiskott-Aldrich syndrome protein
WAVE	Wiskott-Aldrich syndrome protein family verprolin homologs
WMI	White matter injury
µM	Micromolar

Abstract

Oligodendrocytes (OL) are responsible for the production of the myelin sheath involving the neuronal axons, providing nutritional support and fastening the transmission of electrical signals. Oligodendrogenesis impairment or myelin damage, either during neurodevelopment or later in life, may result in impaired brain functions leading to cognitive and/or motor deficits. Increased levels of S100B have been described in several conditions associated with both OL and myelin damage, being associated with a poorer disease prognosis. Moreover, S100B toxic effects have been mainly correlated with engagement of its receptor for advanced glycation end-products (RAGE), which is also highly expressed under these conditions. Thus, the major goal of this thesis was to decipher the involvement of the S100B-RAGE axis in the inflammatory-associated myelin damage, both during neurodevelopmental white matter damage, and upon demyelinating disorders.

First, we showed that excessive S100B levels impaired oligodendrogenesis, either in primary cultures of OL, or in maturating organotypic cultures of cerebellar slices. Indeed, high S100B levels impaired OL differentiation and promoted morphological maturation arrest in pure cultures that was corroborated by deficient myelination in cerebellar slices. Additionally, S100B also compromised neuronal and synaptic integrity, while inducing astrogliosis, nuclear factor (NF)-κB activation and inflammation. Interestingly, the use of the RAGE antagonist FPS-ZM1 prevented S100B effects, indicating that RAGE engagement is necessary for this S100B detrimental role during neurodevelopment. We next observed that demyelination of mature organotypic cultures of cerebellar slices with lysophosphatidylcholine (LPC) markedly enhanced the expression of S100B and RAGE. In parallel, there was an elevation of markers of immature oligodendrocytes, compromised neuronal networks, and a marked gliosis with augmented inflammatory response also via NF-κB induction. Once again the use of the RAGE antagonist FPS-ZM1 prevented LPC effects, suggesting that S100B-RAGE axis have as well an injurious action during demyelination. Finally, we observed that the S100B-RAGE axis was highly expressed in the *in vivo* animal model of multiple sclerosis, the experimental autoimmune encephalomyelitis. These animals, besides showing a chronic elevated clinical score also displayed a great loss of myelin, downregulation of OL and synaptic specific markers, in addition to a potentiated expression of proinflammatory cytokines and inhibition of anti-inflammatory response. Interestingly, when the animals where treated with dimethyl fumarate (DMF), a drug used in clinical practice for multiple sclerosis treatment, although no changes were observed in their clinical score, there was a mild improvement in their cognitive performance. In addition, we observed a marked improvement in myelination, a restoration of oligodendrocyte and synaptic markers, as well as a reduction of inflammation, including a decreased expression of S100B and RAGE.

Our studies imply a toxic role of S100B-RAGE axis in oligodendrogenesis, either during myelin formation, or following demyelination, along with impaired neuronal and synaptic integrity, glia activation and establishment of an inflammatory milieu. Most importantly, this work supports that S100B-RAGE interaction may constitute a new and more specific target for therapeutic

intervention strategies to reduce brain injury associated with inflammation-associated myelin diseases.

Keywords: Biomarker; Demyelinating diseases; Myelin; Oligodendrogenesis; S100B/RAGE axis; Therapeutic strategy

Resumo

Os oligodendrócitos (OL) são as células do sistema nervoso central responsáveis por produzir a bainha de mielina que envolve os axónios, dando-lhes suporte nutricional, protegendo a sua integridade e contribuindo para uma rápida condução dos potenciais de ação, importantes para as funções cognitiva e motora. Ao longo do seu desenvolvimento, os OL apresentam diferentes estádios de acordo com as suas capacidades migratória, proliferativa, de maturação e diferenciação. Para que os OL sejam capazes de mielinizar os axónios existentes na vizinhança, estas células sofrem diversas alterações morfológicas associadas à sua correta maturação, à produção de elevadas quantidades de proteínas da mielina e à extensão de prolongamentos em direção aos axónios. Desta forma, danos na mielina e/ou erros ao longo da oligodendrogénese poderão induzir danos cerebrais e afetar tanto a função cognitiva como a motora, de acordo com o que se verifica em patologias do neurodesenvolvimento – doenças peri- e neonatais, ou da vida adulta, sendo a mais comum a esclerose múltipla (EM).

Curiosamente, neste tipo de patologias têm sido descritas uma expressão e libertação excessiva da proteína S100B, que estão também relacionadas com um pior prognóstico. Apesar de concentrações fisiológicas desta proteína terem um efeito benéfico e serem essenciais para as células gliais e para os neurónios, níveis elevados produzidos em resposta a um dano ou infeção, em paralelo com um microambiente inflamatório, têm sido descritos como tóxicos para estas células. Diferentes estudos têm associado estes efeitos tóxicos à interação da proteína com o recetor de produtos finais de glicação avançada (RAGE), sugerindo que a via S100B-RAGE está de algum modo envolvida no início e/ou progressão de doenças inflamatórias associadas à mielina.

O trabalho experimental desenvolvido ao longo desta tese focou-se em entender o papel da proteína S100B no desenvolvimento e dano dos OL e da mielina, e se esta seria dependente da interação com o seu recetor RAGE. Assim, pretendeu-se avaliar:

(i) o efeito de diferentes concentrações de S100B, mimetizando uma situação fisiológica e patológica, na oligodendrogénese durante a fase de proliferação e no início da diferenciação, e se a ligação ao RAGE modula estes efeitos;

(ii) se os níveis excessivos de S100B, libertados aquando da indução da desmielinização num modelo *ex vivo* de culturas organotípicas do cerebelo, contribuem para a patogénese da mielina, assim como para os mecanismos a ela associados, incluindo a resposta inflamatória, comprometimento da integridade neuronal e sináptica, bem como se a inibição do recetor RAGE previne tais efeitos nefastos;

e (iii) se num modelo *in vivo* de EM – a encefalomielite autoimune experimental, EAE – ocorre igualmente um aumento da expressão do eixo S100B-RAGE em associação aos outros marcadores da doença, e se estes são modulados pelo tratamento com dimetil fumarato (DMF), fármaco de uso clínico na EM.

Inicialmente demonstrámos que concentrações reduzidas de S100B não afetam o desenvolvimento dos OL, como demonstrado pela ausência de alterações no número de células $NG2^+$ e MBP^+, assim como da expressão génica de marcadores específicos de OL em diferentes

estádios de desenvolvimento. Contrariamente, níveis elevados desta proteína induziram uma redução marcada do número de células maduras MBP^+ e da expressão de marcadores específicos de OL maduros, em paralelo com um aumento do número de células precursoras $NG2^+$ e da expressão de marcadores específicos de células precursoras de oligodendrócitos (OPC), levando à inibição da diferenciação dos OL e da sua maturação morfológica. Através do uso de um antagonista específico do RAGE, o FPS-ZM1, mostrámos que os efeitos tóxicos dos níveis excessivos de S100B estavam relacionados com a interação deste com o seu recetor RAGE, uma vez que este preveniu o bloqueio da oligodendrógenese e promoveu a correta maturação das células MBP^+, essencial para o início da mielinização. Estes resultados foram comprovados num modelo *ex vivo* mais complexo, as culturas organotípicas de cerebelo em maturação, mimetizando o período de neurodesenvolvimento, permitindo perceber o papel do eixo S100B-RAGE no processo de mielinização. Neste modelo mostrámos que níveis elevados de S100B leva a um atraso na oligodendrogénese resultando numa deficiente mielinização e num comprometimento da integridade neuronal e sináptica. Em paralelo, ocorreu igualmente a indução da reatividade astrocitária, comprovada pela marcada expressão de GFAP e alteração morfológica dos astrócitos, e a ativação do fator de transcrição NF-κB, levando a uma resposta inflamatória. Curiosamente o uso do FPS-ZM1 preveniu todos estes efeitos comprovando o papel nefasto de concentrações não fisiológicas de S100B no desenvolvimento de OL e formação de mielina numa situação inflamatória do neurodesenvolvimento, através de processos dependentes da interação com o seu recetor RAGE.

Seguidamente, procedemos à indução de desmielinização em culturas organotípicas de cerebelo já maduras, com recurso à lisofosfatidilcolina (LPC), de forma a mimetizar uma situação de desmielinização no adulto. Neste modelo, verificámos uma expressão aumentada de S100B e RAGE, assim como um marcado aumento de marcadores de OL imaturos e células $NG2^+$, em paralelo com uma diminuição de marcadores de OL maduros e células MBP^+. Adicionalmente, a indução de desmielinização levou à redução da área total de neurónios e da expressão de marcadores sinápticos, resultando na perda da integridade neuronal e sináptica. Neste modelo observámos ainda a ativação de células astrocíticas e microgliais, confirmada pelo aumento da expressão das proteínas GFAP e Iba1, respectivamente, assim como pela alteração morfológica das células gliais. Esta ativação glial resultou num aumento da expressão de citocinas pro-inflamatórias, em resultado da indução da via do NF-κB, promovendo a resposta inflamatória. Mais uma vez, o co-tratamento com FPS-ZM1 preveniu os efeitos causados pelos níveis elevados de S100B, suportando o facto de que concentrações elevadas de S100B contribuem para a patogénese da desmielinização através de mecanismos dependentes do RAGE.

Por fim, os estudos num modelo *in vivo* de EM permitiram-nos demonstrar que a proteína S100B e o seu recetor RAGE eram expressos em concentrações basais nos animais controlo e que após indução da EAE, os seus níveis se encontravam aumentados, voltando a níveis inferiores durante o tratamento dos animais com DMF. Adicionalmente, verificámos que a indução de EAE levava a uma grande perda de mielina e redução da função cognitiva, associadas com uma diminuição da expressão génica de marcadores específicos dos OL e de

proteínas sinápticas. Em paralelo observámos ainda uma ativação astrocitária, através do aumento de expressão de GFAP e da alteração morfológica das células $GFAP^+$, com sobre-expressão de citocinas pro-inflamatórias mas sub-expressão de citocinas anti-inflamatórias. Em oposição, a administração de DMF, embora não prevenindo a manifestação de paralisia motora da EAE, preveniu não só a indução da via S100B-RAGE, como também a disfunção cognitiva, a perda de fibras mielinizadas e de expressão de proteínas sinápticas, a ativação astrocitária e a consequente resposta pro-inflamatória.

Em suma, os resultados obtidos nesta tese apontam para um papel nefasto do eixo S100B-RAGE nos mecanismos associados a patologias peri-natais e à EM incluindo: o dano e/ou perda dos OL e/ou mielina, a inibição dos mecanismos de reparação, a perda e/ou disfunção neuronal e sináptica, bem como a ativação glial e o aparecimento de um ambiente neuroinflamatório. Tendo em conta que a expressão de S100B e de RAGE aumenta em resposta ao dano, mas que está diminuída na recuperação, este trabalho aponta para o seu potencial uso como biomarcadores de diagnóstico e prognóstico, assim como de monitorização da progressão da doença e da eficácia da terapêutica. Mais importante, acreditamos que a via S100B-RAGE constitui um novo e mais específico alvo para estratégias terapêuticas neuroprotetoras com o intuito de reduzir o dano cerebral associado a patologias inflamatórias e desmielinizantes, ao mesmo tempo que favorece a reparação.

Palavras-chave: Biomarcador; Doenças desmielinizantes; Estratégia terapêutica; Oligodendrogénese; Mielina; S100B/RAGE

Chapter I

GENERAL INTRODUCTION

1. Oligodendrocytes and myelin

The term oligodendrocyte (OL) means a cell with a few branches, with the origin from the Greek words *oligo* (few), *dendro* (branches) and *cytes* (cells). In the central nervous system (CNS), these cells correspond to approximately 5 to 10% of the total glia population and their primary role is the synthesis and wrapping of layers of myelin – myelination – around neuronal axons (Bunge et al. 1962). This provides electrical insulation, effectively lowering the capacitance and increasing the resistance of the axonal membrane (Bunge 1968).

Myelin has a unique biochemical composition with high content of sphingolipids, cholesterol and myelin-specific proteins. Proteolipid protein (PLP) and myelin basic protein (MBP) are the two most abundant myelin proteins, constituting together about 80-90% of the total myelin proteins (Aggarwal et al. 2011). Although myelination initiates in late embryonic development, it occurs mainly during postnatal life until late adolescence, contributing to the maturation of functional circuits (Zuccaro and Arlotta 2013). Moreover, there is evidence of plasticity of myelin in the adult CNS in response to changes in neural activity, suggesting that myelination may increase in adult life in some regions of the CNS (Emery 2010). Therefore, myelination is of critical importance to the correct development, maintenance and function of the brain.

1.1. Oligodendrogenesis: from birth to myelination

Oligodendrocyte precursor cells (OPC) arise from neural stem cells in different regions of the embryonic neural tube as a response to signals that modulate the activity of several factors, including the Olig genes. Along development, these OPC migrate through CNS and proliferate extensively in response to growth factor expression including platelet-derived growth factor (PDGF) (Yang et al. 2011). Ultimately, OPC differentiate and maturate in order to properly myelinate adjacent axons (Bansal et al. 1989, Lubetzki et al. 1991).

1.1.1. Origin and specification

Along oligodendrogenesis, OPC are generated after neuronal differentiation in sequential waves from specific germinal regions within different parts of the brain and spinal cord (SC) during embryogenesis and early postnatal life (Altman and Bayer 1984).

In the developing mouse SC, the first wave of OPC occurs in the ventricular zone, within the motor neuron precursors domain, which also gives rise to motor neuron precursors, around the embryonic day (E) 12.5 (Pringle et al. 1996). OPC emerge in response to the sonic hedgehog homolog signalling that acts through activation of Olig2, a transcription factor essential for OL development (Lu et al. 2000). Subsequently, a second wave of OPC is generated from a more dorsal progenitor domain (dP3-6) around E15.5 (Cai et al. 2005, Fogarty et al. 2005). Lately, a third wave starts after birth, but its specific origin remains unclear (Rowitch and Kriegstein 2010).

In the developing mouse forebrain, OPC arise in a similar pattern. Around E12.5, an initial wave of OPC develops from Nkx2.1-expressing precursors in the medial ganglionic eminence and closely associated to the anterior entopeduncular area of ventral telencephalon (Olivier et al. 2001, Spassky et al. 2001). Subsequently, these ventrally-derived OPC migrate to populate all

parts of the telencephalon, entering the cerebral cortex at E18. A second and third wave of OPC arise from the lateral and caudal ganglionic eminences at E15.5 and from the cortex after birth, respectively, giving rise to the majority of adult OL (Kessaris et al. 2006). Once initial glial fate is achieved and as a result of arising from highly restricted progenitor domains, OPC exhibit a multidirectional migration in the ventricular zone to distant sites under control of several repulsive and attractive cues (Miller 2002).

1.1.2. Proliferation, differentiation and myelination

The progression along oligodendroglial lineage occurs through distinct differentiation steps that can be identifiable accordingly to their migratory capacity, morphological complexity and the expression pattern of specific markers (Bansal et al. 1989, Lubetzki et al. 1991). During maturation, oligodendroglial cells suffer architectural changes and acquire a complex branched morphology, losing their capacity to proliferate and migrate. In this context, there are four identified stages of OL differentiation: OPC, late OPC or preOL, immature or pre-myelinating OL and mature or myelinating OL (Wigley et al. 2007); although it may also be identified a transient stage between mature and myelinating cells, the mature non-myelinating OL (Baumann and Pham-Dinh 2001), as detailed in Figure I.1.

Initially, OPC have a high proliferative and migratory capacity and a specific expression of PDGF receptor α (PDGFRα), the ganglioside A2B5, the transcription factor Olig2, the proteoglycan neural-glial antigen 2 (NG2) (Pringle et al. 1992, Nishiyama et al. 1996), the polysialic acid-neural cell adhesion molecule (Grinspan and Franceschini 1995), among others. At this stage, OPC are usually characterized by a small polygonal soma, with few processes (Chittajallu et al. 2004). During the differentiation process, OPC give rise to preOL that extend multipolar short processes and start to express, in addition to OPC markers, the sulfatide O4 antibody (Sommer and Schachner 1981), which expression persists until the immature OL stage. Terminal differentiation into post-mitotic pre-myelinating OL requires profound changes in both cellular behaviour and gene expression. In fact, immature post-mitotic OL present long ramified branches (Gard and Pfeiffer 1989, Armstrong et al. 1992) and loose the expression of immature markers (A2B5, NG2 and PDGFRα). Besides the continuous expression of O4, they start to express a more mature marker O1 antibody, which binds to galactocerebroside C (Yu et al. 1994). Moreover, terminal differentiation and subsequent myelination are tightly coupled events, with the intermediate pre-myelinating OL representing a highly transient stage wherein cells either rapidly progress to myelination or undergo apoptosis (Barres et al. 1992, Barres et al. 1992, Trapp et al. 1997). For the initial step of myelin formation, immature OL need to extend several cytoplasmic protrusions (filopodia) in order to find suitable myelin-competent axons. Lately, mature OL synthesize a high levels of myelin proteins in an orderly manner, such as MBP, PLP, myelin associated glycoprotein (MAG), and finally myelin OL glycoprotein (MOG). In parallel, OL extend membranes that form compact enwrapping myelin sheaths around the axons (Reynolds and Wilkin 1988, Scolding et al. 1989, Zhang 2001). As OPC differentiate, the changes in expression

of molecular markers and cell morphology are also accompanied by a loss of synaptic connections with neurons and a change in ion channel expression (Kukley et al. 2010).

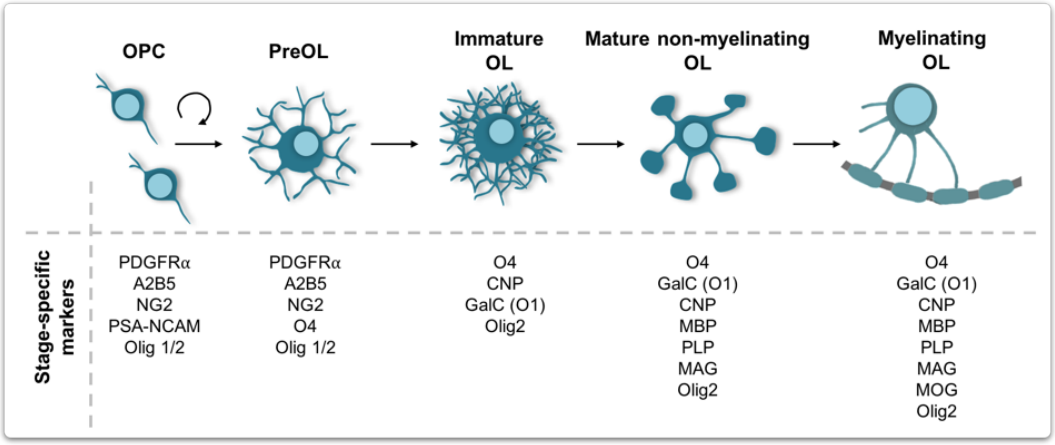

Figure I.1. Schematic representation of the different oligodendrocyte stages during oligodendrogenesis. These stages are identifiable accordingly to their increasingly complex morphology, the expression pattern of well-defined markers and their ability to proliferate, migrate and differentiate. CNP, 2',3'-cyclic nucleotide 3'-phosphodiesterase; GalC, galactocerebroside C; MAG, myelin associated glycoprotein; MBP, myelin basic protein; MOG, myelin oligodendrocyte glycoprotein; NG2, neural-glial antigen 2; PDGFRα, platelet-derived growth factor receptor α; PLP, proteolipid protein; PSA-NCAM, polysialic acid-neural cell adhesion molecule.

1.1.3. Oligodendrogenesis temporal line: human *vs*. rodent

Human brain development is a complex process that starts in the fourth week of gestation and extends throughout the lifespan (Stiles and Jernigan 2010, Silbereis et al. 2016). More specifically, OL development begins during the second trimester of gestation and progresses towards birth, and further into adulthood (Back et al. 2001, Jakovcevski and Zecevic 2005). In humans, it was also described the existence of the same four successive OL stages between 10 and 40 gestational weeks (Figure I.2.). The first OPC are observed in the forebrain at 10 weeks, although higher OPC numbers are found only around 15 weeks, when they are most numerous in the ganglionic eminences and in the cortical subventricular zone (Jakovcevski et al. 2009). Throughout the last half of gestation $NG2^+/O4^+/O1^+$ late progenitors are the predominant oligodendroglial cells. Between 18 and 28 weeks, OPC and preOL are the most predominant OL cells, nevertheless a minor population of $O4^+/O1^+$ immature OL (9.9±2.1% of total OL) was also identified. Later, between 28 and 41 weeks, an increase in immature OL (30.9±2.1% of OL) is accompanied by a progressive increase in MBP^+ cells, initially restricted to the periventricular white matter and first detected by immunocytochemistry around 30 weeks (Kinney 2005). Around the 30 week it is observed a dramatic increase in multipolar $O4^+$ cells mostly in deeper and milder cerebral white matter, while they are sparsely distributed in the superficial white matter, and not detected in the cortex. Lately, MBP^+ cells are extensively found between 36 and 40 weeks, with an increase from 1 to 5% in total brain volume that contains myelinated white matter (Huppi et al. 1998, Back et al. 2001).

In rodents, OPC are first observed at the telencephalon, around E9.5 (Timsit et al. 1995). In accordance, another study clarified that in E13, ventral telencephalic regions have a greater capacity to generate OL *in vitro* than the corresponding cortical regions (Birling and Price 1998).

In addition, PDGFRα mRNA in the mouse brain was first detected at E15 (Yeh et al. 1993, Yeh et al. 1993). In contrast, at P2 preOL compose the majority of OL stage in the cerebral white matter with a lower number of immature OL. In parallel, mRNA for MBP was detected later, at P3 and P4 in the mouse corpus callosum and the internal capsule, respectively (Verity and Campagnoni 1988). At P7 the white matter contains more than 80% immature OL, that start the myelination process (Dean et al. 2011). Finally, MBP expression reaches the peak in the mouse cortical and subcortical white matter at P20, when primary myelination is completed (Campagnoni and Macklin 1988, Verity and Campagnoni 1988).

In an overall evaluation human *vs* rodent oligodendrogenesis seems similar between the two species (Figure I.2). In the human forebrain, the first early OPC appear at 10 weeks, which can be compared to E9.5 in mice. The P2 rodents seems to correspond to the period between 18 and 28 weeks in human, in which preOL and few immature OL compose the cerebral white matter, whereas the white matter at P7 from rodents presents a state of maturation similar to the one observed in humans between 30 and 36 weeks (Craig et al. 2003). Finally, the first MBP^+ cells are observed around P7 rodents, becoming increasingly abundant at P14 (Hartman et al. 1979, Bjelke and Seiger 1989), what is similar to the initial myelination in humans around 36 to 40 weeks and the increased extent of myelination at full-term infants (Dean et al. 2011).

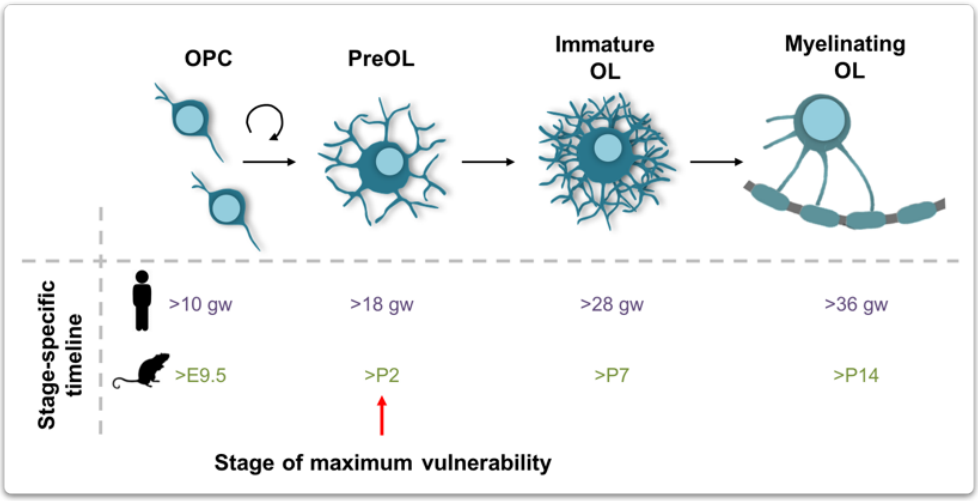

Figure I.2. Schematic comparison of oligodendrogenesis temporal line between human and rodents. Oligodendrocyte precursor cells (OPC) appear around 10 gestational weeks (gw) in humans and around embryonic day (E) 9.5 in rodents. Oligodendrocyte (OL) lineage between 18 and 28 gw in humans is similar to that of at postnatal day (P) 2 in rodents and is composed mainly by OPC and preOL, with a minor population of immature OL. White matter between 28 and 30 gw in humans is similar to that observed at P7 rodents, with predominance of immature oligodendrocytes and a progressive increase in mature OL. Whereas mature OL start to be abundant around 36 gw in humans and, similarly, at P14 in rodents.

1.1.4. Myelin function and repair: myelination, demyelination and remyelination

The final stage of OL development – myelination – occurs predominantly in the postnatal period and is a multi-step process that involves the recognition and adhesion of OL to the appropriate axon, the synthesis and transport of myelin components to the OL outer membrane, the wrapping of the myelin membrane around the axons and the compaction of the myelin sheath (Baumann and Pham-Dinh 2001, Sherman and Brophy 2005, Nash et al. 2011) (Figure I.3.). OL

myelinate the axons that have a diameter of at least 0.2 μm, but not the axons with a lower diameter that usually remain unmyelinated (Waxman and Bennett 1972, Voyvodic 1989). Furthermore, the number of layers in the myelin sheath is tightly correlated with the axonal diameter, increasing in proportion to the inner axonal diameter. Each OL has the capacity to myelinate up to 50 axons simultaneously (White and Krämer-Albers 2014), influencing neuronal size and axon diameter (Tzakos et al., 2005; Witt and Brady, 2000). These segments of myelinated axon – internodes – are delimited by the areas of naked axons where action potentials are generated – nodes of Ranvier – (Nualart-Marti et al. 2013). Therefore myelination promote rapid conduction of the action potential, enhancing the integration of information across spatially distributed neural networks supporting cognitive and motor functions (O'Rourke et al. 2014). Neuronal function is further influenced by factors secreted by OL that induce sodium channel clustering, required for potentiation of the conduction along axons, which is maintained even in the absence of direct axon-glial contact (Baumann and Pham-Dinh 2001, Bradl and Lassmann 2010). In addition, OL synthesize a number of trophic factors including nerve growth factor (NGF), neurotrophin-3, brain-derived neurotrophic factor (Dai et al. 2003), glial cell-line derived neurotrophic factor, and insulin-like growth factor 1 (IGF-1) (Wilkins and Compston 2005, Smith et al. 2013) that contribute to neuronal survival and to the maintenance of axonal integrity (Blank and Prinz 2014, Simons et al. 2014). Also myelin proteins may participate in neuronal protection. Indeed 2',3'-cyclic nucleotide 3'-phosphodiesterase (CNP) protein present in non-compact myelin (Gravel et al. 2009), although not essential for myelination, is required for long-term axonal integrity (Lappe-Siefke et al. 2003) due to its ability to convert 2′,3′-cyclic adenosine monophosphate (cAMP) to 2′-adenosine monophosphate, promoting adenosine production (Beirowski 2013). Therefore, myelination is of critical importance for neuron survival and integrity, by providing them protection and nutritional support (Mei et al. 2013), as well as for synaptogenesis by improving conduction of action potentials (Franklin and Ffrench-Constant 2008).

Notwithstanding, in a disease situation it may occur OL dysfunction and/or death as well as myelin injury, which result in the demyelination of axons and consequent impairment of neurons and respective nerve impulse transmission. Demyelination is usually followed by spontaneous remyelination that activates myelin repair mechanisms and induces the production of new myelin. A successful remyelination requires the proliferation and migration of OPC to the lesion site, and their correct differentiation and maturation into myelinating OL (Miron et al., 2011). This process enables the restoration of the saltatory conduction of nerve impulses and the maintenance of the necessary conditions for axon survival. Nonetheless, with the exposure to repeated demyelinating insults, remyelination efficiency decreases and fails to restore axon function. Remyelination failure may be associated to patient age, a toxic environment in lesions (e.g. cellular debris and inflammation), and/or errors in migration, proliferation and differentiation of OPC (Chandran et al., 2008; Franklin and Ffrench-Constant, 2008; Patel and Klein, 2011).

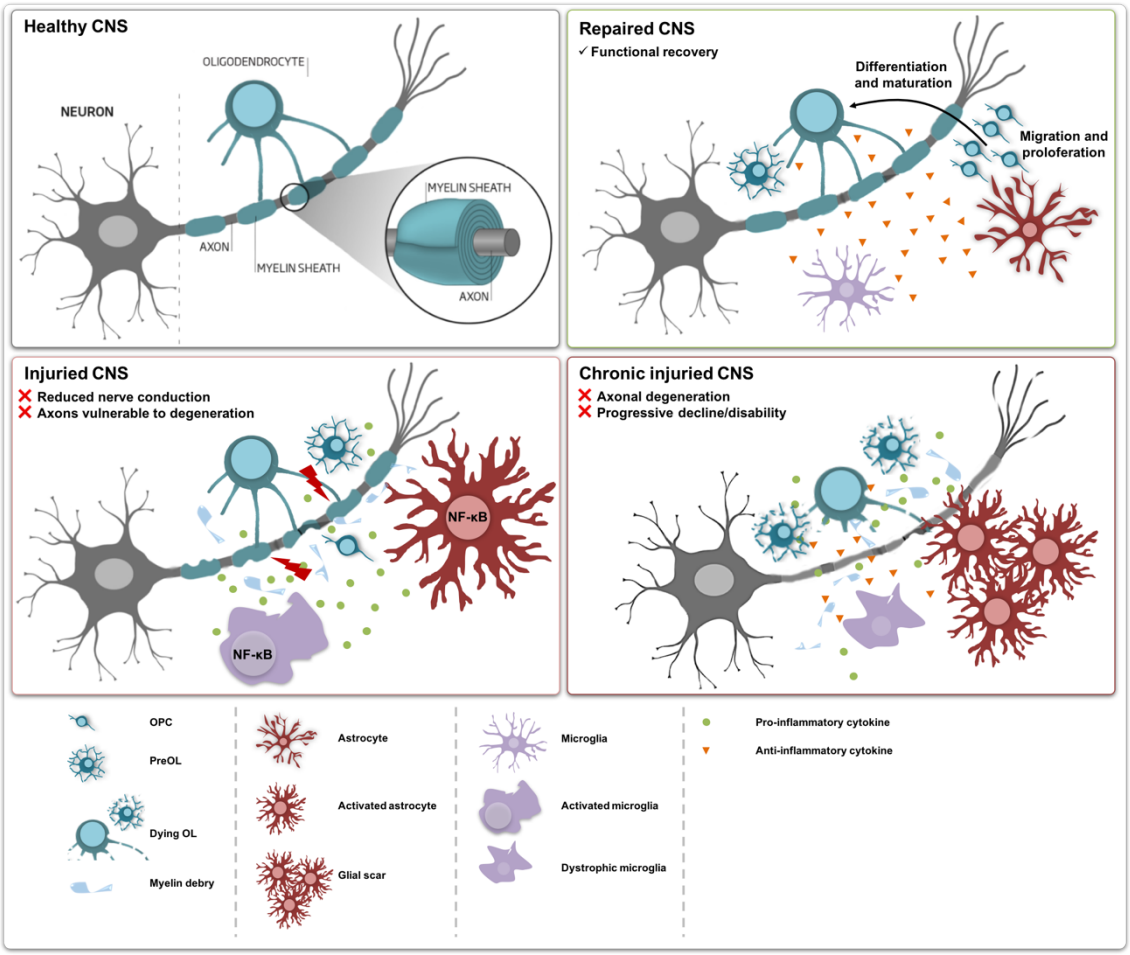

Figure I.3. Schematic representation of injury and repair processes in the central nervous system. In a healthy brain, oligodendrocytes (OL) synthetize a myelin membrane that enwraps the axons and contributes to neuronal survival and maintenance of axonal integrity. In a disease situation, OL and/or myelin injury result in the demyelination of axons and consequent impairment of proper neuronal function. Demyelination is usually followed by spontaneous remyelination that requires the proliferation and migration of oligodendrocyte precursors cells (OPC) to the lesion site, as well as a correct differentiation and maturation into myelinating OL. Myelination enables the maintenance of the necessary conditions for axon survival. Nonetheless, with the disease progression and exposure to repeated demyelinating insults, remyelination efficiency decreases and fails to restore axon function, culminating in neurodegeneration.

1.2. Intrinsic cues in myelination, demyelination and remyelination

The dynamic nature of OL to extend processes, to sense and myelinate axons require a complex and dynamic system of signalling mechanisms, like those from transcription factors or cytoskeleton components.

1.2.1. Transcription factors

Transcription factors have been implicated in the regulation of OL development, from lineage specification through their progressive stages of maturation until myelination (Gokhan et al. 2005; Stolt et al. 2006). The best-studied families of transcription factors are the Olig genes (Olig1 and Olig2) and Sox group (Sox2, Sox8, Sox9 and Sox10) (Liu et al. 2007). Regarding the Olig genes, although structurally similar and expressed co-ordinately, they encode proteins with distinct biological roles, being responsible for either promoting or inhibiting OPC differentiation

(Balabanov and Popko 2005, Ligon et al. 2006). Olig2 is necessary for oligondendroglial cell specification and differentiation. Indeed, Olig2 appears to be the only one absolutely required for the specification of the lineage, with Olig2 null mice displaying a near complete loss of PDGFRα$^+$ and NG2$^+$ OPC (Lu et al. 2002, Copray et al. 2006, Ligon et al. 2006). Interestingly, Olig2 is strongly upregulated during acute brain damage (Buffo et al. 2005), indicating a possible increase in OPC proliferation to counteract defects in the number of OL associated with a demyelinating process. More recently, it was shown that Olig2 recruits the chromatin-remodeling enzyme BRG1 to regulatory elements of key genes during differentiation, including Sox10, Smad-interacting protein-1 (Sip1) as well as pro-myelination transcription factors such as myelin regulatory factor (MRF) (Cahoy et al. 2008). On the other hand, Olig1 appears to be mostly implicated in OL maturation, being involved in the final stages of myelin production (Lu et al. 2002, Xin et al. 2005) and regeneration (Arnett et al. 2004). In fact, Olig1 signalling is essential for the transcription of myelin proteins and its absence leads to OL arrest at an early stage of differentiation (Xin et al. 2005). Interestingly, the Olig1Cre-neo$^{-/-}$ generates some pre-myelinating OL with delayed myelination. However, Olig1Cre$^{-/-}$ mice exhibits loss of myelinating OL and severe demyelination deficits leading ultimately to animal death (Xin et al. 2005). Additionally, as OL mature, Olig1 translocation from the nucleus to the cytoplasm is required to promote full membrane extension (Niu et al., 2012). Thus, indicating that Olig1 signalling might not be required for the initial stages of OL differentiation, but for their subsequent correct maturation and axonal myelination. Moreover, nuclear translocation of Olig1 during the remyelination phase promotes myelin repair as described after demyelination induction by myelin toxicants such as lysolecithin (or α-lysophosphatidylcholine, LPC) or cuprizone (Arnett et al. 2004, Burton 2005).

Concerning the Sox family, it is known that Sox9 is involved in OL specification since generation of OPC is substantially delayed in Sox9 conditional knockout embryos (Stolt et al. 2004). In Sox8-deficient mice, OL development proceeds normally until birth, nonetheless terminal differentiation of OL is transiently delayed at early postnatal phase (Stolt et al. 2004). Sox10 null mice exhibit a similar, but more severe phenotype with a relative normal number of OPC and a complete loss of mature OL expressing MBP and PLP (Stolt et al. 2002). This implicates both Sox 8 and Sox10 as being necessary for the development of myelin-forming OL (Stolt et al. 2004). Moreover, Sox10 induces myelination together with the pro-myelination factor MRF, which is specifically and rapidly induced upon OL differentiation ahead of myelin genes such as PLP and MBP (Emery et al. 2009). Indeed, Sox10 has a direct activity on MRF induction since its expression is almost completely lost in the absence of Sox10 (Hornig et al. 2013). Therefore, in the absence of MRF the generation of OPC and the initial differentiation into pre-myelinating OL is unaffected. However these pre-myelinating OL fail to upregulate the majority of myelin genes and to myelinate, undergoing instead into apoptosis (Emery et al. 2009). Moreover, Sox10 mutations or ablation has been linked to dysmyelinating and demyelinating diseases (Ito et al. 2015). Also Sox2 have been implicated in oligodendrogenesis, since during developmental myelination Sox2 expression is reduced in the later stages of OL lineage associated with myelination. Studies *in vitro*, demonstrated that Sox2 overexpression promotes rat OPC

proliferation and inhibits differentiation. In contrast, Sox2 knockout resulted in decreased OPC proliferation, impaired differentiation and survival, suggesting that Sox2 contributes to the expansion of OPC during the recruitment phase of remyelination (Zhao et al. 2015). In fact, *in vivo* studies following toxin-induced demyelination in mice with inducible loss of Sox2 revealed reduced OPC recruitment within the lesion and a less efficient differentiation into remyelinating OL, resulting in impaired remyelination (Zhao et al. 2015).

In addition to these well-established transcriptional regulators of OL differentiation, a number of new players have emerged. Zfp488, an OL-specific zinc finger transcription factor, promotes OPC formation in the presence of Notch signalling and OL differentiation in the presence of Olig2. Moreover, Zfp488 overexpression promotes remyelination after cuprizone-induced demyelination enhancing the OL differentiation (Soundarapandian et al. 2011), while Zfp488 knockdown decreases myelin gene expression (Wang et al. 2006), suggesting that Zfp488 also plays a role in promoting myelination and remyelination.

Sip1 [also named zinc finger homeobox protein 1b or Zeb2] is enriched in differentiated OL but weakly expressed in OPC, while its expression is significantly downregulated in Olig1- and Olig2-null mice. Sip1 knockout mice exhibit a severe failure of myelin sheath formation without affecting OPC proliferation, indicating that Sip1 is required for the transition of OPC to myelinating OL and consequently to myelination (Weng et al. 2012). Curiously, in mutant Sip1 mice, deficient Schwann cells (SCs) failed to fully differentiate and were unable to sort and myelinate peripheral nerve axons (Quintes et al. 2016). Also Sip1 deletion arrested SCs at an undifferentiated state and re-maturation of Sip1-deficient SCs into remyelinating cells was dramatically hampered after nerve injury (Quintes et al. 2016, Wu et al. 2016), suggesting that Sip1 may also be involved in CNS remyelination.

1.2.2. Cytoskeleton

Along their development, OL are under continuously cytoarchitecture alterations in order to form and extend their processes. Such changes in OL morphology are in part mediated by two major cytoskeletal components: microtubules (MT) and microfilaments (MF) (Bauer et al. 2009). Both components are expressed in different degrees and within distinct regions at all stages of differentiation, where MT confer mechanical stability to OL processes and MF mediate process outgrowth and basic stability. To begin myelin sheath formation, immature post-mitotic OL first need to extend numerous cytoplasmic protrusions (filipodia), in order to find suitable myelin-competent axons. As MF-rich filipodia extend, they are invaded by MT, thus further enlarging these processes and converting them to lamellipodia (Mullins et al. 1998, Svitkina and Borisy 1999, Bretschneider et al. 2004). To accommodate this rapid directed growth, the OL cytoskeleton increases MF polymerization and branching.

In OL, MF exist as globular monomers (G-actin) or as filamentous polymers (F-actin). Interestingly, in OL processes edge are found numerous F-actin assembly and remodeling proteins, such as the actin-related protein 2/3 complex, Wiskott-Aldrich syndrome protein (WASP) family proteins, myosin II, and the small Rho GTPases (Rac1, Cdc42, and RhoA) (Song et al.

2001). WASP proteins are controlled by the Rho family GTPases, which mediate actin polymerization (Begum et al. 2004).

Active Rac1 and Cdc42 are positive regulators of morphological differentiation, inducing process extension and branching, through the activation of neuronal (N)-WASP and Wiskott-Aldrich syndrome protein family verprolin homologs (WAVE) 1/2 (Derivery and Gautreau 2010). Subsequently, these proteins interact with the actin-related protein 2/3 complex to generate branched F-actin networks at the cell's leading edge (Ridley 2011). Indeed, WAVE1-deficient OL form fewer processes and those formed lack lamellipodia (Kim et al. 2006). Similarly, chemical inhibition of N-WASP leads to defects in OPC process extension, and filopodia and lamellipodia retraction, in parallel with an impaired axonal ensheathment (Bacon et al. 2007).

In contrast, RhoA acts as a negative regulator inhibiting process elongation through the activation of its downstream effector Rho-associated kinase (ROCK) (Liang et al. 2004). RhoA/ROCK's activates myosin II, which in turn, leads to actomyosin contraction (Wang et al. 2012). On contrary, differentiation is triggered through downregulation of RhoA/ROCK's activity, which loosens contractile forces and allows the OL to extend processes and eventually form the membrane (Bauer et al. 2009, Wang et al. 2012). Moreover, Fyn activation block the inhibitory actions of RhoA (Wolf et al., 2001; Taniguchi et al., 2003; Kippert et al., 2007), consequently, leading to hyperextension of OPC processes and increased branch formation. Fyn activation also triggers tyrosine phosphorylation of focal adhesion kinase, which is crucial for the activation of Cdc42/Rac1 (Hoshina et al. 2007).

MT are composed by heterodimers of α- and β-tubulin protein subunits (Lunn et al. 1997, Simpson and Armstrong 1999). With the OL maturation and the increased complexity, MT undergo several posttranslational modifications, including α-tubulin acetylation, which is correlated with higher stability of MT at the extensions (Lunn et al. 1997, Song et al. 2001), and the maintenance of the dense branched network (Bauer et al. 2009). In this sense, in order to increase MT dynamics during OL maturation and myelination, tubulin must be continuously deacetylated (Li et al. 2007). MT associating and/or binding proteins are also present in the OL (Bauer et al. 2009). One of the more intriguing is the CNP, a canonical myelin protein required for process outgrowth, since it binds tubulin heterodimers and drives MT assembly (Lee et al. 2005). CNP overexpression leads to exuberant process outgrowth and myelin production, followed by myelin compaction failure (Gravel et al. 1996, Yin et al. 1997). Therefore, an equilibrium in CNP levels is needed for myelin growth and compaction in order to achieve a stable membrane.

1.3. Extrinsic cues in myelination, demyelination and remyelination

Neurons, astrocytes and microglia have been implicated as playing a critical role in several aspects of myelination including the modulation of OL activity, myelin maintenance, clearance of myelin debris, and myelin renewal (Iacobas and Iacobas 2010, Moore et al. 2011, Miron et al. 2013, Skripuletz et al. 2013). These cells release factors that provide OL support and regulate their development, such as growth factors, chemokines and cytokines. Likewise, the final number of total OL may be determined by the competition for limiting amounts of these factors.

Nevertheless, prolonged and increased release of inflammatory mediators, like cytokines, may mediate deleterious effects, ultimately resulting in the onset of demyelinating diseases. In addition, upon injury an exacerbated and sustained inflammatory milieu may also have a negative impact in the repair and remyelination processes, leading to the progression of the disease.

1.3.1. Growth factors

Many growth factors have been found to be involved in the OL development, through the regulation of their migration, proliferation and differentiation ability. In addition, some of these growth factors are also expressed during demyelination and implicated in remyelination, including PDGF, fibroblast growth factor (FGF) and IGF-1.

Both PDGF and FGF-2 act as potent chemoattractants and/or as chemokinetic molecules, stimulating OPC motility (Mitew et al. 2014). In addition, PDGF is a potent mitogen, synthesized during development by both astrocytes and neurons, essential for OPC survival and proliferation, preventing premature differentiation (Noble et al. 1988, Raff et al. 1988). In fact, overexpression of PDGF results in a marked increase in the number of OPC (Calver et al. 1998), while in PDGF knockout mice the number of OPC is dramatically reduced (Fruttiger et al. 1999). PDGF is also involved in remyelination, increasing OL density and reducing apoptosis after chronic demyelination (Vana et al. 2007).

FGF-2 stimulates early and late OPC proliferation, by upregulating Olig2 and PDGFRα expression. This results in the arrest of their subsequent differentiation and maturation into mature OL (McKinnon et al. 1990, Bansal and Pfeiffer 1994), confirmed by the downregulation of the major myelin proteins, at both the protein and mRNA levels (Goddard et al. 2001). Curiously, FGF has been reported to also regulate the myelin sheath thickness of CNS axons. Indeed, FGFR1/2 knockout did not affect the number of myelinating OL but instead the myelin sheath thickness. Furthermore, FGFR1/2 absence in adulthood causes a lower transcription of myelin genes and the formation of disproportional myelin sheath thickness relative to the axon diameter (Furusho et al. 2012). Concerning FGF-2 role in demyelination/remyelination, studies report contradictory data. On the one hand, it was reported that FGF-2 significantly inhibits the clinic pathological signs of experimental autoimmune encephalomyelitis (EAE), being associated with a significant reduction in the numbers of myelin-toxic cells and a consecutive increase in myelin-producing OL and OPC in the proximity of demyelinated lesions (Ruffini et al. 2001). In accordance, FGF-$2^{-/-}$ mice display a reduced remyelination extent (Rottlaender et al. 2011). On the other hand, other studies showed that activation of the FGF-2 receptor results in the inhibition of myelin production (Goddard et al. 2001), increased FGF-2 levels trigger demyelination in the adult rat brain (Butt and Dinsdale 2005) and that the lack of FGF-2 is associated with OL regeneration (Armstrong et al. 2006).

IGF-1 promotes OPC survival, proliferation and myelination. IGF-1 and IGFR-1 knockout mice exhibit a decrease in myelin protein expression and a reduction in the number of both OPC and mature OL (Zeger et al. 2007), while opposite effects were observed upon IGF-1 overexpression. Moreover, IGF-1 also acts synergistically with FGF-2 and PDGF to promote OPC proliferation

(McMorris et al. 1990). After cuprizone-induced demyelination, IGF-$1^{-/-}$ mice exhibit impairments in OPC proliferation and survival, as well as, inadequate remyelination (Mason et al. 2000, Mason et al. 2000, Mason et al. 2003). In addition, in transgenic mice continuously expressing IGF-1, OL are protected from apoptosis during cuprizone intoxication (Mason et al. 2000), suggesting a beneficial role of IGF-1 in remyelination.

Several other growth factors including neurotrophin-3 (Barres et al. 1994), CNTF (Barres et al. 1993), brain-derived neurotrophic factor (Van't Veer et al., 2009) and neuregulin (Roy et al., 2007; Brinkmann et al., 2008; Taveggia et al., 2008) also act as mitogens to increase OPC proliferation and survival.

1.3.2. Cytokines

Cytokines are pleiotropic factors that mediate inflammatory responses that promote pathogen clearance and prevent excessive tissue damage (Banyer et al. 2000). Although, proinflammatory cytokines overproduction may lead to excessive inflammation and cell death, anti-inflammatory cytokines exhibit critical roles in repair mechanisms. Some cytokines are involved in OL development and myelin-related processes, such as tumor necrosis factor (TNF)-α and interleukin (IL)-1β. In physiological concentrations, TNF-α and IL-1β induce the release of other growth factors, which in turn regulate OL maturation, repair, regeneration and remyelination (Merrill 1992). However, pathological levels have been implicated in several demyelinating disorders, and may contribute to OL death and demyelination. In fact, both cytokines are able to induce neurotoxicity through elevated glutamate production resulting in excitotoxic death (Takahashi et al. 2003, Ye et al. 2013) or OL death in a calcium dependent manner (Sherwin and Fern 2005).

TNF-α signals through tumor necrosis factor receptor (TNFR)1 and TNFR2, activating two different pathways: one that leads to apoptosis via death mediated caspase activation (TNFR1) and another that activates survival and protective pathways via nuclear factor kappa β (NF-κβ) (TNFR1 and TNFR2) (Barnhart and Peter 2003, Micheau and Tschopp 2003). Non-physiological concentrations of TNF-α were shown to be directly toxic to OL (Robbins et al. 1987, Merrill et al. 1993) and to myelin (Selmaj and Raine 1988). The recombinant TNF-α injection into the optic nerve (Jenkins and Ikeda 1992) and on myelinated cultures of mouse spinal cord tissue (Selmaj and Raine 1988) was described to induce demyelination. Moreover, cuprizone-induced demyelination in TNF-α and TNFR2 knockout mice resulted in a significant delay in remyelination (Arnett et al. 2001) due to a reduction in the pool of proliferating OPC and mature OL. Further studies using mice lacking TNFR1 or TNFR2 confirmed that TNFR2, but not TNFR1, is necessary for OL maturation and regeneration (Arnett et al. 2001, Kircik and Del Rosso 2009).

IL-1β was shown to exert cytotoxic effects on mature OL *in vitro* (Merrill 1991, Brogi et al. 1997) and the apoptosis of OL in co-culture with astrocytes and microglia (Takahashi et al. 2003). Perinatal exposure to IL-1β resulted in loss of mature OL and impaired myelination (Fan et al. 2010), whereas a stereotaxic intracerebral injection significantly reduced the number of developing OL (Cai et al. 2004). Other study also demonstrated that mice exposed to IL-1β presented long-lasting myelination defects characterized by a significant reduction of the density

of myelinating OL accompanied by an increased density of OPC and a disrupted expression of several transcription factors known to control OL maturation, suggesting a partial blockade in the OL maturation process (Favrais et al. 2011). Regardless cuprizone-induced demyelination in IL-1β knockout mice do not affect the severity of demyelination, and the number of OPC and mature OL within demyelinating lesions (Mason et al. 2001), these animals fail to adequately remyelinate due to lack of IL-1β-mediated expression of IGF-1 (Mason et al. 2001, Mason et al. 2003). Accordingly, co-administration of IL-1 receptor antagonist with lipopolysaccharide reduced brain injury by improving myelination (Pang et al. 2003). Curiously, IL-1β inhibited OPC migration through its receptor IL-1 receptor type 1, whereas IL-1 receptor antagonist treatment and IL-1 receptor type 1 knockout had no effect on the OPCs proliferation, but did promote the recruitment of newly generated OPCs to the corpus callosum and improved remyelination (Zhou et al. 2017). So it seems that IL-1β may be needed for functionally remyelination.

Besides, both TNF-α and IL-1β increase gliosis (Cai et al. 2004), and together with other cytokines, can stimulate local inflammatory cytokine production (Benveniste and Benos 1995, Liu et al. 1998). These proinflammatory cytokines can also cause blood- brain barrier (BBB) breakdown (Quagliarello et al. 1991, Blamire et al. 2000) allowing a further amplification of the systemic inflammatory response and exacerbating the overall inflammatory environment.

1.3.3. Chemokines

Chemokines induce directed chemotaxis in responsive cells, to guide them to sites of injury. Many chemokines including CXCL12 and CXCL1/CXCL2 are induced during CNS development and direct cell migration and maturation (Tsai et al. 2002, Stumm and Hollt 2007), suggesting that these molecules could also have a role in CNS repair.

CXCL12 have a directed role in OPC chemotaxis, however, their maturation into OL is associated with increased CXCR7 expression and decreased CXCR4 (Dziembowska et al. 2005, Maysami et al. 2006, Gottle et al. 2010). In fact, *in vivo* CXCL12 stimulation was shown to promote morphological maturation of OPC and myelin protein expression, whereas pharmacological inhibition of the CXCR7 blocked the CXCL12-dependent effects in the EAE model (Gottle et al. 2010). Curiously, OPC differentiation is correlated with the downregulation of CXCR4 (Dziembowska et al. 2005). In agreement, using the cuprizone model of remyelination, CXCR4 inhibition, via pharmacological blockade or siRNA technology, led to the arrest of OPC maturation by preventing the expression of myelin proteins (Patel et al. 2010). Moreover, in CXCR4-defective mice, OPC migration was decreased in response to CXCR4 antagonist and anti-CXCL12 antibody (Dziembowska et al. 2005). These data suggest that the CXCL12-CXCR4 axis may be important in the recruitment, proliferation and differentiation of OPC during remyelination of the adult CNS.

CXCL1 and CXCL2 play a role in inflammation, oligodendroglial biology and myelin disorders through the interaction with their receptor CXCR2 (Charo and Ransohoff 2006). In the viral JHMV model, the expression of the chemokines CXCL1 and CXCL2 and CXCR2 was increased within the spinal cord during the chronic infection phase. Interestingly, treatment with CXCR2 antagonist

resulted in increased numbers of apoptotic cells, associated with elevated expression of cleaved caspase 3 and muted Bcl-2 expression, mainly within white matter tracts of the spinal cord and delayed clinical recovery (Hosking et al. 2010). In transgenic mice overexpressing CXCL1 during EAE induction, it was observed a decrease in clinical severity correlated with a decreased demyelination and increased remyelination (Filipovic et al. 2003). In disagreement, in demyelinating *ex vivo* studies, CXCR2 blockage led to enhanced myelin repair. Also in EAE and during cuprizone intoxication studies, $CXCR2^{+}$ neutrophils contributed to demyelination (Liu et al. 2010, Liu et al. 2010), whereas systemic injection of a CXCR2 inhibitor at the onset of EAE decreased the number of demyelinated lesions (Liu et al. 2010). In addition, $CXCR2^{-/-}$ chimeric mice presented earlier and potentiated OPC proliferation in the demyelinated lesions, comparing with $CXCR2^{+/+}$ animals (Liu et al. 2010, Liu et al. 2010). These data provide support for a dual role of these chemokines and its receptors in the regulation of OL development in myelin-associated processes.

1.4. Models to assess myelination, demyelination and remyelination

As abovementioned, oligodendrogenesis and subsequent related processes are of critical importance. Therefore, the evaluation of such processes and underlying mechanisms are required in order to better understand which factors and cells are involved in OL proper development or their responses to numerous stimuli. In this context, several experimental approaches can be used depending on the question addressed (Figure I.4.).

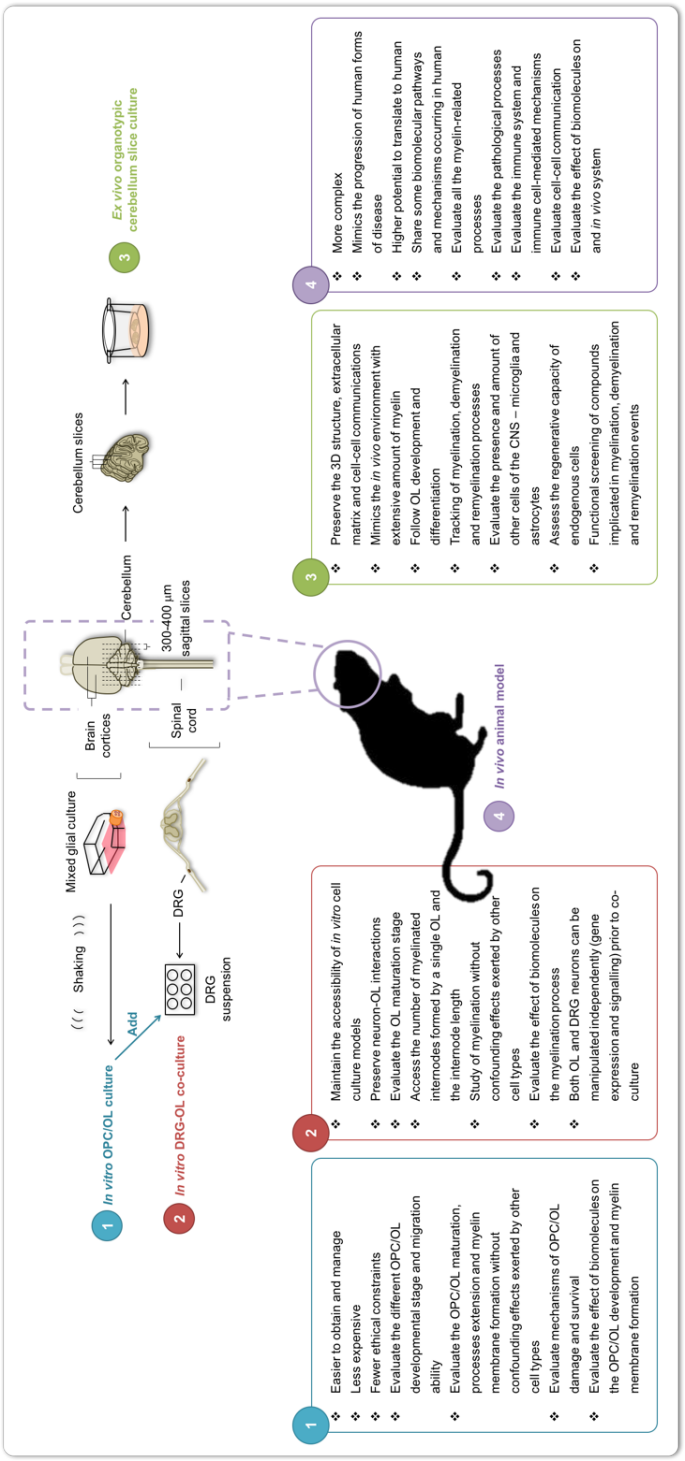

Figure I.4. Schematic representation of different models to assess myelin-related processes. (**1**) To assess specific oligodendrocyte (OL) functions/mechanisms we may use primary OL cultures. Here, OL are obtained from dissociated rodent cortex that is plated on flasks in order to obtain mixed glial cultures. After culture growth, OL are isolated by shaking and maintained in culture with media supplemented with proliferation factors to obtain primary oligodendrocyte precursor cell (OPC) culture or in the absence of these factors to obtain primary mature OL cultures. (**2**) To study *in vitro* myelination it may be used OL-dorsal root ganglia (DRG) neuron co-cultures. OL are obtained as in (1), added to DRG neurons isolated from rodent embryonic spinal cord and maintained in culture to allow myelination. (**3**) To assay myelination in a tissue context we may use organotypic cerebellar slice cultures. These cerebellar slices are obtained from rodent brain and cultured on an air-liquid interface. (**4**) Finally, several animal models are available to evaluate myelination changes in several central nervous system (CNS) regions, taking in consideration the interactions between the CNS and the peripheral system.

1.4.1. *In vitro* oligodendrocyte culture

In vitro approaches are crucial to study the role of CNS cells, allowing manipulations that cannot easily be performed *in vivo*. The isolation and culture of OPC (McCarthy and de Vellis 1980) can be useful to understand the mechanisms involved in the development of myelin and to study the effect of drugs on the migration, survival, proliferation and differentiation of these cells (Chen et al. 2007, Dincman et al. 2012). Thus this *in vitro* model represents a first step in ascertaining the effects of either treatments or the manipulation of a molecular pathway during myelination. Moreover, since the cells are not influenced by other types of cells or the environment, results are more easily interpreted (Merrill 2009).

After OPC isolation from mixed glial cultures, these cells are maintained in a proliferative stage by factors such as PDGF-A and FGF-2 that allow these cells to continuous proliferate (Bogler et al. 1990, Noble et al. 1990). On the other hand, if the proliferating factors are removed from the culture medium and differentiation inducers such as thyroid hormone are added, OPC will differentiate into mature OL able to synthesize membrane sheets whose organization and composition are very similar to that of myelin (Szuchet et al. 1986).

One major drawback of OL monoculture systems is the lack of neuron-OL interactions. In order to maintain the accessibility of *in vitro* cell culture models, myelinating cultures can also be achieved by co-culturing purified populations of neurons and OPC that have been independently prepared (Merrill 2009). These types of cultures allow the evaluation of the myelination process in a more defined system without the interference of the effects exerted and factors released by other glial cells. The source of neurons is usually the dorsal root ganglia, which neurons are cultured on 3D matrixgel and maintained in the presence of NGF and of fluorodeoxyuridine to inhibit mitoses of non-neuronal cells, being lately added the OL following growth factors removal (Stevens et al. 2002, Ishibashi et al. 2006, Zhang et al. 2006). Dorsal root ganglia neurons extend their neuritis radially from their somas forming a dense neurite network (O'Meara et al. 2011). This fact facilitates the observation and analysis of myelination when compared with neurons dissociated from CNS that are very difficult to analyse due to its reticular distribution (Thomson et al. 2006).

1.4.2. *Ex vivo* organotypic slice cultures

Organotypic slice cultures preserve the three-dimensional architectural organization that supports all tissue circuits and cell-cell interactions allowing the study of several neurobiological processes, making them an intermediate model between cell cultures and animal models. When the main goal is to evaluate changes in myelination, organotypic cerebellar slice cultures (OCSC) are usually used, which allow the evaluation of OL maturation process and myelination in a well-defined region (Suzuki et al. 2012). In fact, myelination in cerebellum occurs postnatally and OCSC from early postnatal animals replicate the *in vivo* myelination process (Jaeger et al. 1988, Notterpek et al. 1993). Therefore OCSC provide a model system for studying myelination, demyelination, and remyelination (Mi et al. 2009, Miron et al. 2010, Huang et al. 2011), as well as

to evaluate the link between neurodegeneration and inflammation (Aktas et al. 2005, Dorr et al. 2005).

Parasagittal slices (250-400 µm) are prepared from P7 rat cerebellum, maintained in culture on semiporous membranes and kept at an air-liquid interface, which causes less tissue thinning and allow much simple maintenance and processing (Stoppini et al. 1991, Lossi et al. 2009). After 7 days in vitro (DIV), is already evident a high degree of myelination with significant number of myelin segments aligned with axons. Before any treatment slice cultures should be kept for 3 DIV in culture to allow the clearance of debris and the stabilization of cerebellar slices. If the goal is to study the effect of a stimulus on myelin formation and/or *de novo* myelination, the culture should be treated around 3-4 DIV, before myelination is complete, and the system analysed at 7 DIV. However, if the purpose is to evaluate the effect of an insult on demyelination and/or remyelination, the slices must be kept for 7 DIV prior to treatment and then analysed after treatment or during the next 14 DIV, where remyelination usually takes place (Miron et al. 2010). Demyelination is currently induced by exposure to LPC, a detergent that mainly destroys myelin (Birgbauer et al. 2004). After the transient demyelinating insult, OPC proliferate in response to demyelination and differentiate into mature OL that remyelinate. Similar to *in vivo* remyelination, regenerated myelin in OCSC is thinner with shorter internodes (Zhang et al. 2011, Madill et al. 2016), recapitulating a normal remyelination course. These observations indicate that OCSC reasonably recapitulate much of the complex process of true myelin regeneration.

1.4.3. *In vivo* animal models

By using *in vivo* animal models is possible to preserve the brain circuits and evaluate changes in myelination at different CNS regions. Besides, is also possible to evaluate changes in the systemic circulation and their effects in the CNS, being very important for the study of diseases that are initiated at the systemic level and have a secondary CNS phenotype or CNS disorders that will progress and be exacerbated following immune cell infiltration (Osorio-Querejeta et al. 2017).

1.4.3.1. Models of virus-induced inflammatory demyelination

Among the most commonly used models for the induction of myelin-associated neurological diseases are mouse hepatitis virus (MHV) and Theiler's murine encephalomyelitis virus (TMEV). TMEV is a natural pathogen of mice, causing paralysis and encephalomyelitis in a wide range of mouse strains (Mecha et al. 2013). Besides, it also induces clinical neurological disease in immunocompetent mice, associated with brain and spinal cord atrophy (van der Star et al. 2012). Thus, TMEV offers the advantage of examining the impact of therapies in the course of the clinical disease. Following intracranial injection, the virus induces myelin damage in the grey matter disease that is followed by viral spread and persistence in the white matter. The timing and outcome of these events are dependent on the mouse strain (Mecha et al. 2013).

The MHV was first isolated from a mouse with hind limb paralysis, presenting disseminated encephalomyelitis and prominent demyelination (Bailey et al. 1949). Demyelination is induced

when MHV is inoculated intracraneally or intranasally (Bender and Weiss 2010), with the prominent demyelination plaques associated with clusters of activated microglia/macrophages. There is evidence that latent infection occurs in glial cells, since after intracranial injection, viral replication is observed in astrocytes, microglia, and OL during the demyelinating phase (Baker and Amor 2015).

1.4.3.2. Models of toxic demyelination

Toxic demyelination models enable the study of de- and remyelination processes, since alterations in the CNS due to inflammatory processes driven by adaptive immunity are absent. The most frequently used model is induced by systemic exposure to the copper-chelator cuprizone (Gudi et al. 2014, Praet et al. 2014). Cuprizone ingestion induces highly reproducible demyelination of certain brain regions, among them the corpus callosum. Cuprizone causes dysfunction of mitochondrial complex IV and is selectively toxic to mature OL (Venturini 1973, Komoly et al. 1987), inducing OL apoptosis and demyelination through mechanisms of oxidative injury. Cuprizone feeding is usually performed for ~5 weeks to induce acute demyelination, and upon withdraw there is a rapid and extensive remyelination (Hiremath et al. 1998). However, if chronic demyelination is induced, with ~12 weeks of cuprizone intake, myelin repair is significantly decreased (Matsushima and Morell 2001).

Other toxic models include the focal injection of LPC (Hall 1972, Jeffery and Blakemore 1995) or ethidium bromide into specific white matter tracts including the corpus callosum (Blakemore 1982). LPC induces demyelination due to a direct action of the toxin on the myelin sheath (Hall and Gregson 1971, Jeffery and Blakemore 1995). It is a highly reproducible model of primary demyelination, with the advantage of triggering a focal lesion in a defined region within the CNS. As in other toxic models of demyelination, the phase of myelin destruction is rapidly followed by remyelination (Lassmann and Bradl 2017).

1.4.3.3. Models of experimental autoimmune encephalomyelitis

Currently, one of the most widely applied model in multiple sclerosis (MS) research is the EAE, in which autoimmunity to CNS components is induced in susceptible mice through immunization with self-antigens derived from myelin proteins such as MBP, PLP and MOG (Rivers et al. 1933) – actively induced EAE – together with a Freund's adjuvant and pertussis toxin that potentiate the humoral immune response and induces BBB disturbance, respectively, inducing oscillatory or chronic symptoms similar to that found in MS patients (Tuohy et al. 1989). However, remyelination is not very extensive perhaps due to the dense infiltration of macrophages and microglia in the lesion over prolonged periods of time. The only indication of impressive levels of remyelination in EAE is observed in focal models of cortical demyelination involving immunization with subclinical doses of MOG in parallel with injection of proinflammatory mediators (Merkler et al. 2006).

Alternatively, EAE can be induced following adoptive transfer of lymph node cells, or specific T cell lines and clones (both $CD4^+$ and $CD8^+$) from immunised animals –passive EAE. Whatever the induction regimen, initially during the acute phase it occurs the infiltration of mononuclear cells

in the CNS, that can be followed by a recovery period referred as remission – chronic relapsing EAE. In the relapse phase myelin damage and axonal loss is more prominent than in the acute stage (Kipp et al. 2012). An additional advantage of chronic relapsing EAE mouse is the development of secondary progressive disease, with these mice showing extensive demyelination, axonal and neuronal loss, in parallel with a marked gliosis, all features of progressive MS (Al-Izki et al. 2011).

The lack of an effective therapy for the treatment of demyelinating diseases to date as well as the poor understanding of its correct pathophysiology led to the development of experimental models that mimic both the symptoms and the disease hallmarks. Besides the existence of all these different models, the heterogeneity of myelin-associated diseases is very difficult to mimic and there is still no perfect model. Nevertheless, the current models allow the highlighting of several crucial mechanisms that may underlie the onset and progression of the demyelinating diseases.

2. Myelin-related disorders

The fundamental importance of proper myelin development and homeostasis is highlighted in myelin related diseases, in which not only the conduction velocity of nerve impulses is reduced, but also naked axons are vulnerable to degeneration (Franklin and Ffrench-Constant 2008). Since OL are responsible for myelin production, their pathophysiology is emerging as an essential event in the occurrence of such disorders.

Myelin-related disorders can be divided into two large groups, dysmyelinating diseases, defined by inappropriate production of myelin, or demyelinating diseases characterized by OL damage and total or partial loss of myelin that compromises neuronal proper function and integrity. The OL and myelin injury occurs in response to intrinsic or external factors, such as inflammatory processes, metabolic derangements, hypoxia-ischemia and viral infections, that might target either the own myelin or the cells responsible for synthesizing it (Love, 2006; Mayo et al., 2012). Usually, following demyelination spontaneous remyelination might occur allowing a partial recovery. It is the balance between demyelination and remyelination that defines the outcome of the disease (Franklin and Ffrench-Constant, 2008). CNS dysmyelinating diseases are usually associated with neurodevelopment while demyelinating disorders can appear during peri- and neonatal development or in adulthood, being MS the most common in later developmental stages (Bunyan et al., 2012; Popescu and Lucchinetti, 2012).

2.1. Peri- and neonatal white matter injury

Brain injury in the premature infant is associated with a high risk of neurodevelopmental disability and it primarily involves cerebral white matter damage since the principal target are the developing OL. In fact, recent evidences have been linking myelin abnormalities and white matter injury (WMI), described during the peri- and neonatal periods, to impaired OL maturation resulting thereby in their reduced capacity to synthesize proper myelin (Tolcos et al. 2017). During these stages of brain development the white matter damage is potentiated, since vulnerable OPC and

pre-myelinating OL are the predominating cells of the OL lineage that are present (Back et al. 2001). Although largely associated with premature birth, WMI may also commonly occur in some groups of full-term infants where *in utero* insults appear to coincide with a susceptible period in white matter development prior to the onset of cerebral myelination (Back 2017).

Based on clinical, epidemiologic and experimental studies, WMI occurrence and severity involves a wide variety of pre- and perinatal factors including genetic factors, or exposure to extrinsic insults such as hypoxia-ischaemia, hypothyroxinaemia, intrauterine infection, postnatal sepsis, inflammation, drug and toxicant exposures, pain, neonatal stress and malnutrition (Saliba and Marret 2001, Back and Rosenberg 2014).

2.1.1. Peri- and neonatal white matter injury types

During human cerebral development, WMI is a common lesion that can vary considerably in severity and three major forms of pathology are described: focal cystic necrosis, focal microscopic necrosis, and diffuse non-necrotic lesions (Figure I.5.).

The most severe form of WMI involves large regions of cystic necrosis that typically ranges from about 1 to 6 mm in diameter (cysts), whereas mid-necrosis results in microcysts typically measuring less than a millimeter (Back 2014). The large cysts are considered the hallmark of periventricular leukomalacia (PVL) and are commonly found in the deep white matter. However, in its most severe forms, PVL can extend into the centrum semiovale and even the subcortical white matter (Pierson et al. 2007). Cystic necrosis rapidly culminates in death of all cellular elements (glia, axons, blood vessels, and neural progenitors) due to severe energy failure. As necrosis progresses to the focal infiltration, there is an activation of immune cells (macrophages and microglia), including lipid-laden macrophages, in parallel with an early and progressive astrogliosis that delineates the borders of focal necrosis. Similar to focal cystic necrosis, microcysts involve tissue destruction and are enriched in cellular debris, degenerating axons and increasing the density of phagocytic macrophages (Back 2017). In both necrotic WMI, there is an almost complete myelination failure as a consequence of the degeneration of all cellular elements.

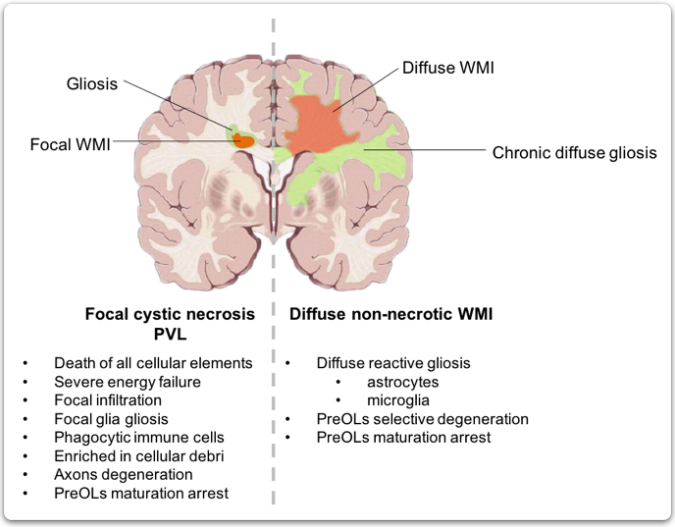

Figure I.5. Schematic representation of perinatal white matter injury categorization. Focal white matter injury (WMI) indicates white matter necrosis with pancellular degeneration leading to depletion of white matter glia and axons. This

severe necrosis results in cystic periventricular leukomalacia (PVL), whereas milder necrosis results in microcysts with selectively early preOL death. Chronic lesions are enriched in reactive glia (astrocytes and microglia/macrophages), which generate inhibitory signals that block preOL differentiation to mature myelinating OL. In diffuse WMI myelination failure results from preOL arrest rather than axonal degeneration. The molecular mechanisms that trigger preOL arrest are likely to be multifactorial and related to factors intrinsic and extrinsic to the preOL.

On the other hand, diffuse WMI is the most common form of injury described in contemporary cohorts of preterm newborns and it may exist isolated or be associated with foci of necrosis (Pierson et al. 2007). In contrast to necrotic injury, diffuse WMI is defined by a chronic diffuse reactive gliosis comprising activated astrocytes and microglia and a selective degeneration of preOL, triggered by oxidative stress and other insults, whereas axons are mostly spared in pre-myelinating white matter except when necrotic foci are present (Haynes et al. 2008, Riddle et al. 2012). In the early stages of injury, numerous reactive microglia are present in the periventricular white matter, whereas reactive astrocytes are a prominent feature of later lesions (Back et al. 2007).

2.1.2. Pathophysiology of peri- and neonatal white matter injury

Whereas in earlier times preterm infants were at high risk for destructive white and grey matter degeneration, preterm survivors now commonly display less severe injury. Nevertheless, these milder forms of injury are associated with reduced cerebral growth that involves disrupted repair mechanisms resulting in abnormal and failed maturation of both neurons and glia rather than in cell death during the critical window of neural circuitry development.

The last weeks of gestation and the first postnatal months are crucial periods for white matter maturation, being this period highly vulnerable to any kind of insult. Several cellular and molecular mechanisms, as inflammatory factors, have been implicated in pre-myelinating OL injury and death (Back and Rosenberg 2014), resulting in myelin defects. Impaired myelination during the perinatal period will consequently result in axon dysfunction and abnormal electrical conduction, therefore contributing to brain loss of function and long-term neurological impairments including neurodegenerative conditions.

2.1.3. Demyelination and neurodegeneration in peri- and neonatal injury

PreOL are the predominant OL lineage cells in the white matter during the high-risk period for WMI and are the primary cell type to suffer in early diffuse WMI upon exposure to hypoxia-ischemia or inflammation, culminating in impaired myelination. Studies show that despite an extensive reduction in the number of early myelinating OL in acute lesions, the density of preOL (Segovia et al. 2008) and the entire population of OL (Billiards et al. 2008) are actually increased rather than decreased in chronic lesions. These results confirm that alterations in myelination in injured white matter occurs not due to OL degeneration, but because the progress of oligodendroglial cell maturation becomes stalled, mainly at a pre-myelinating stage of OL development. This may be explained by the fact that developing white matter is highly populated by OPC resistant to hypoxia-ischemia that proliferate extensively in response to preOL

degeneration, resulting in their rapid expansion (Back 2015). However, these newly generated preOL display arrested differentiation in chronic lesions and fail to myelinate despite the presence of numerous intact-appearing axons (Billiards et al. 2008). This maturation impairment may negatively influence subsequent white matter correct development and axonal functional integrity.

Concerning mature neurons, neuronal degeneration is a more prominent feature of necrotic WMI lesions that often contain dystrophic axons and axonal spheroids. Indeed, it was observed significant neuronal loss in human cortex, subplate, basal ganglia, thalamus and cerebellum in necrotic WMI but not in autopsy samples of diffuse WMI (Pierson et al. 2007). Therefore, neuronal loss may directly occur from primary energy failure or secondary axonal degeneration triggered by necrotic WMI. In contrast, neither immature grey matter neurons nor white matter axons displayed significant oxidative injury or degeneration (Back et al. 2005), rather do fail to mature correctly (Riddle et al. 2012). Immature neurons were shown to respond aberrantly and display widespread disturbances in maturation of their dendritic arbors and synapses, which further contributes to impaired cerebral growth and abnormal synaptic activity. Interestingly, the numbers of spines are reduced in dysmature dendrites of projection neurons in both cortex (Dean et al. 2013) and caudate (McClendon et al. 2014) in a prenatal cerebral ischemia model.

Thus, cellular dysmaturation in grey and white matter may disrupt a critical developmental window that coincides with rapid brain growth and enhanced neuronal connectivity related to elaboration of the dendritic arborization, synaptogenesis, and myelination.

2.1.4. Inflammation in peri- and neonatal injury

Both experimental and clinical studies associated inflammatory mediators with WMI. Inflammation may disrupt white matter development through (in)direct toxicity to OL or their precursors, inhibiting OPC differentiation (Cammer and Zhang 1999), stimulating OL apoptosis (Selmaj et al. 1991, Louis et al. 1993), and causing myelin degeneration (Probert et al. 1995). Activated microglia release cytokines, such as TNF-α and interferon-γ (IFN-γ), toxic to oligodendroglial cells (du Plessis and Volpe 2002). This toxicity is synergistic and appears to involve mediation by other glia. In fact, astrocytes modify the mechanisms of microglial-mediated TNF-α toxicity (Li et al. 2008). Recent data suggest that cytokine toxicity may be mediated through disturbances in glutamate transport, since TNF-α and IL-1β impair glutamate transporter function and potentiate glutamate-mediated oxidative stress. Conversely, the anti-inflammatory cytokines IL-4 and IL-10 inhibit this effect through an indirect reduction in proinflammatory production (du Plessis and Volpe 2002).

2.1.5. Therapeutic strategies to peri- and neonatal white matter injury

Even with the dramatic changes in management over the last two decades, the rates of neurodevelopmental abnormalities among ex-preterm infants remain high (Hutchinson et al. 2013). In this sense, efforts to minimize injury, preserve growth, and identify interventions focused on antioxidant and cytokine-suppressive anti-inflammatory pharmacological approaches are being explored (Keelan and Newnham 2017). The compound (+)-naloxone, a potent toll-like

receptor 4 signalling antagonist which suppresses immune NF-κB activation and cytokine biosynthesis (Cheng et al. 2014, Wang et al. 2014), was shown to protect against sepsis in animal models (Miller et al. 1986, Law and Ferguson 1988). An alternative approach is the inhibition of IL-1. Pregnant mice exposure to rytvela, a IL-1R antagonist, improved fetal and neonatal outcomes following exposure to either IL-1β or lipopolysaccharide challenge (Quiniou et al. 2008). Indeed, rytvela blocked uterine/placental/intra-amniotic inflammation, and prevented fetal demise, and inflammation-related morbidities (Quiniou et al. 2008, Nadeau-Vallee et al. 2015, Nadeau-Vallee et al. 2017).

In addition, a number of promising neuroprotective agents including corticosteroids, erythropoietin (EPO) and darbepoitin, melatonin and magnesium sulfate are being investigated in animal models and clinical trials (Glass et al. 2015, Davis et al. 2016). In fact, a recent randomized controlled pilot study showed that melatonin administration to neonates with hypoxic-ischemic encephalopathy preserved the serum concentration of superoxide dismutase, reducing the production of nitric oxide, and ameliorated brain injury (Aly et al. 2015). Antenatal corticosteroid administration has been reported to significantly improve the rates of death, intraventricular hemorrhage (IVH), PVL (Carlo et al. 2011) and cerebral palsy (CP), as well as to decrease the risk of severe disability (Sotiriadis et al. 2015). Erythropoiesis-stimulating agents, such as EPO and darbepoetin, resulted in improved cognitive outcomes and reduced CP (Ohls et al. 2014). Besides, EPO treated infants presented reduced brain injury and improved white matter development (Leuchter et al. 2014, O'Gorman et al. 2015). Magnesium sulfate treatment is associated with improved neurologic outcomes, resulting in lower rates of IVH and CP (van de Bor et al. 1987, Crowther et al. 2003, Rouse et al. 2008), as well as, motor dysfunction improvement (Doyle et al. 2009).

Other strategies include the induction of mild hypothermia to term neonates with signs of perinatal asphyxia and encephalopathy, which has been reported to reduce brain injury, the risk of death, and/or major developmental disability at age 18 (Rutherford et al. 2010, Bonifacio et al. 2011). Furthermore, hypothermia increases the rate of survival with normal neurological function (Tagin et al. 2012).

2.2. Multiple sclerosis

MS is a chronic autoimmune inflammatory demyelinating disease. It is considered the most common cause of non-traumatic disability in young adults with an onset occurring usually between the 20 and 40 years, although it has been recently described as increasing during childhood (Cross et al. 2012). Recent data show that MS affects more than 2.5 million people worldwide, of which about 70% are women (Thompson et al. 2008). Prevalence and incidence rates vary with geography and ethnicity, with prevalence ranging from 2/100 000 in Japan to greater than 100/100 000 in Northern Europe and North America. Additionally, MS has a higher prevalence in developed regions (e.g. Europe with 80/100 000) than in underdeveloped countries (e.g. Africa with 0,3/100 000) (WHO, 2008). In Portugal, accordingly to the most recent studies of the

European Multiple Sclerosis Platform, MS affects 7000 persons, of which about 70% are known to receive directed therapy for the disease (Platform , Figueiredo et al. 2015).

Since MS is a complex disorder, several hypotheses have been pointed out as potential causes of the disease. Nonetheless, it is accepted that MS aetiology may result from lifestyle, environmental and genetic factors (Amato et al. 2017). To date, the reported environmental factors with the strongest evidence for involvement in MS include vitamin D deficiency, smoking and Epstein–Barr virus infection (Belbasis et al. 2015). Relying genetic factors, the main genetic susceptibility locus in MS patients resides within the *HLA-DRB1 DRB1*1501* and *DRB1*1503* gene that increases the risk for disease development 2-4 times (Amato et al. 2017). Interestingly, transcription of the HLA-DRB1*1501 locus is regulated by vitamin D (Niino and Miyazaki 2015).

2.2.1. Clinical course and symptomatology

The clinical course and symptomatology of MS (Figure I.6.) are extremely heterogeneous and related with the age of the patients at onset. Yet, although some patients have only minimal symptoms, the majority ultimately develops disability over time as a result of incomplete recovery from relapses and/or conversion to a progressive phase of the disease (Tullman 2013). The heterogeneous presentation is dependent on the brain area affected (Kearney et al. 2015), however the most common symptoms include sensory and visual disturbances, motor impairments, fatigue, pain and cognitive deficits (Compston and Coles, 2008).

The efforts to categorize patients by general patterns of disease presentation have allowed the recognition of 3 main disease subtypes (Lublin 2014). The most common, affecting approximately 85% of patients, is the relapsing-remitting MS (RRMS) form, characterized by an initial episode of neurological dysfunction (clinically isolated syndrome), followed by a remission period within which symptoms improve or disappear and then recurrence of relapse and remission episodes. Clinically isolated syndrome coincides with focal CNS inflammation and demyelination in which myelin, OL and axons become damaged (Hanafy and Sloane, 2011; Peterson and Fujinami, 2007). Whereas relapses are associated with the reactivation of old lesions or even with the appearance of new ones, remittance periods are the result of the resolution of inflammation and remyelination (Chandran et al., 2008; Compston and Coles, 2008). Further, the extent of recovery from a relapse and time range between relapses greatly varies among patients, with the latent phase between the first manifestation of MS and the first relapse going from little months to several years. Eventually, recovery from each episode tends to be incomplete and improvement during each remission wanes with consequent gradual accumulation of axonal degeneration and irreversible neurological deficits (Compston and Coles, 2008; Trapp and Nave, 2008). This situation favours the progression of RRMS to secondary progressive MS (SPMS) in approximately 80% of patients (De Stefano et al. 2001, Fisniku et al. 2008). SPMS is defined by a continuous and irreversible neurological degeneration, with little or absent inflammation and remyelination that are accompanied by CNS atrophy, resulting in decreased brain volume and increased axonal loss (Vukusic and Confavreux, 2003). Lastly, approximately 10% of patients are diagnosed with primary progressive MS (PPMS), which features progressive decline since

disease onset, in parallel with an absence of clinical relapses that correspond to a lesser degree of inflammation and a greater proportion of axonal loss, even during the early disease course (Bashir and Whitaker 1999). This MS form is most commonly diagnosed in individuals who are older than 50 at the time of onset and is more resistant to the medications typically used to treat the disease (Goldenberg 2012, Tullman 2013).

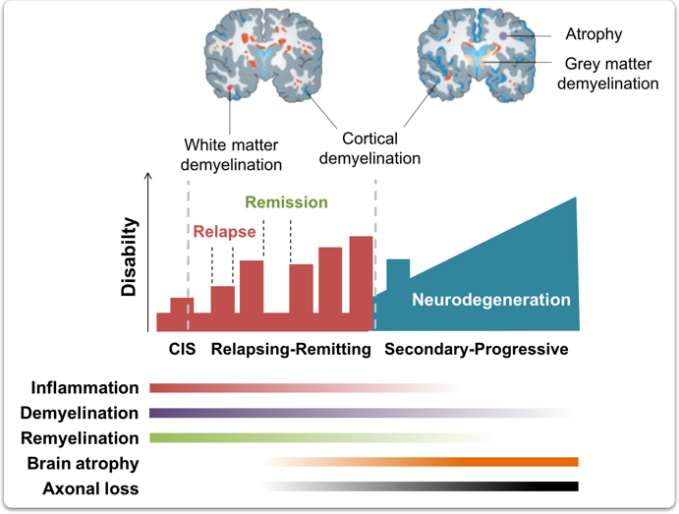

Figure I.6. Schematic representation of multiple sclerosis types and features. The most common form of multiple sclerosis (MS) is the relapsing-remitting MS (RRMS), characterized by an initial episode of neurological dysfunction (clinically isolated syndrome, CIS), followed by a remission period. CIS coincides with focal CNS inflammation and demyelination, whereas relapses are associated with periods of the resolution of inflammation and remyelination. Along time, recovery tends to be incomplete with consequent accumulation of axonal degeneration and brain atrophy, leading to the occurrence of progressive forms.

2.2.2. Pathophysiology of multiple sclerosis

The mechanisms underlying MS include demyelination, axonal/neuronal damage, inflammation, gliosis, oxidative stress and excitotoxicity together with immune system alterations and BBB disruption, followed by spontaneous remyelination (Brassat, 2012; Corthals, 2011). Though the correct order they occur to induce MS pathogenesis is not fully understood.

2.2.3. Demyelination and neurodegeneration in multiple sclerosis

MS is characterized by the occurrence of areas of acute focal inflammatory demyelination, with variable gliosis and relative axonal loss with limited remyelination (Frischer et al. 2009). This situation culminates in the formation of chronic multifocal sclerotic plaques or lesions and extensive axonal injury within the CNS (Calderon et al. 2006). These plaques' location, number, size, and shape vary greatly among MS patients. Plus, these lesions, although disseminated throughout the CNS, occur preferentially in optic nerves, subpial spinal cord, brainstem, cerebellum, and periventricular white matter regions (Compston and Coles, 2008; Love, 2006). MS lesions evolve differently during early versus chronic disease phases, and within each phase, different types and stages of demyelination and neurodegeneration are evident. In line with this, actively demyelinating white matter lesions are defined by the exacerbated infiltration of macrophages and microglial cells and the ongoing destruction of myelin with abundant macrophages containing myelin degradation products. These lesions are more frequently

observed in RRMS patients than in patients with other type of progressive MS (Geurts and Barkhof 2008). In opposite, inactive or chronic active MS lesions are characterized by a rim of microglia and/or macrophages without cytoplasmic myelin protein degradation products, surrounding an almost complete demyelinatied area and severe astrogliosis. These lesions are usually observed in progressive MS (Stadelmann 2011, Stadelmann et al. 2011).

In an attempt to study the heterogeneity of MS pathology related to early and active demyelinating lesions, four neuropathological patterns were determined based on the presence or absence of complement and immunoglobulins, apoptotic nuclei, and/or preferential loss of myelin proteins. In patterns I and II, it is observed T cell and macrophage infiltration and loss of all myelin proteins, leading to demyelination. Pattern II is also defined by additional immunoglobulin and complement deposition along myelin sheaths and within macrophages. Pattern III lesions are characterized by distal oligodendrogliopathy with a preferential loss of MAG and OL apoptosis. Pattern IV lesions are rare and associated with non-apoptotic OL death in the periplaque of non-demyelinated white matter (Metz et al. 2014, Antel et al. 2015).

Apart from the inflammatory demyelination, axonal loss is frequent in acute lesions, supporting the concept that axonal loss occurs at disease onset and is a continuous process. This neuronal degeneration may be related with a post-demyelination increased axonal vulnerability to proteolytic enzymes, matrix metaloproteases, cytokines, oxidative products, free radicals and glutamate (Trapp and Stys 2009, Lassmann 2013). Nevertheless, it was also described that axons itself can suffer a specific immunologic attack, since in acute MS lesion it was found transected axons surrounded by macrophages and activated microglia. Chronic active MS lesions are dominated by tissue destruction with massive loss of both myelin and OL as well as axons, associated with a prominent gliotic reaction and not active inflammation (Dutta and Trapp 2014).

MS lesion can also be located in the grey matter, especially in the cerebral cortex and associated with a progressive course. Cortical lesions have been observed in 80% of patients with PPMS. The histopathological characteristics of these cortical lesions differ substantially from white matter lesions (Calabrese et al., 2010). Three types of cortical lesions have been reported. Two of these lesions may appear in continuity with subcortical white matter plaques (type I) or as small intracranial perivascular lesions (type II). The most abundant form of cortical demyelination is subpial demyelination, which appears as large band-like lesions extending from the outer surface of the cortex into its deeper layer (type III) (Peterson et al., 2001).

2.2.4. Inflammation in multiple sclerosis

Although immune-mediate theory of MS pathology is not the only under discussion, is the most described and has been the target of extensive research (Corthals, 2011; Nakahara et al., 2012). Indeed, inflammation is present at all stages of MS although it is more pronounced in acute phases than in chronic phases.

In line with this, some myelin defects trigger inflammation with consequent axonal degeneration (Nave 2010). Moreover, is also hypothesized that activation of autoimmune responses against myelin components in the CNS occur through a variety of mechanisms such

as molecular mimicry, bystander activation and epitope spreading (Vanderlugt and Miller 2002). Antigen-presenting cells present myelin proteins to CD4+ T cells activating them. Once activated, myelin-specific T cells can cross the BBB with the help of proteases and chemokines (Hauser and Oksenberg 2006), where they proliferate and secrete proinflammatory cytokines (IFN-γ, TNF-α, TGF-β, IL-10 and IL-17), beginning the immune response (Wootla et al. 2012, Tullman 2013). CD8+ T cells also seem to be involved in the pathogenesis, but in contrast with CD4+ T cells, can directly interact with and damage antigen-expressing cells, such as neurons and OL (Stuve et al. 2002). T cells, besides recognizing as the target antigens the myelin associated proteins: MBP (Pette et al. 1990, Valli et al. 1993), MAG, MOG (Zhang and Raus 1994), and PLP (Greer et al. 1997), are also able to stimulate microglia, macrophages and astrocytes, and recruit B cells. In MS patients, the proinflammatory effects of B cells seem to predominate over their anti-inflammatory effects, by secreting antibodies that react specifically with myelin antigens and OL with or without the presence of the complement (O'Connor et al. 2005). Together, these cells produce more inflammatory cytokines and other stressors, including proteolytic enzymes, oxidative products and free radicals (Amor et al., 2010; Van der Walt et al., 2010). The accumulation of these proinflammatory factors as well as the cells that produce them lead to the recruitment of naïve microglia and their consequent activation. This phenomenon will further amplify the inflammatory and immune response (Lassmann and van Horssen, 2011; Nakahara et al., 2012), which ultimately results in myelin, OL and axonal damage, causing irreversible neurological deficits (Hohlfeld 2010).

Accordingly, in histopathological studies, early lesions show invading peripheral immune cells and BBB disruption. Macrophages dominate the infiltrate, followed by CD8+ T cells, whereas lower number of CD4+ T cells, B cells and plasma cells are present. At this phase of the disease, there is little damage to the brain and spinal cord in the areas of normal appearing white matter, although general brain atrophy is already noted (Chard et al. 2002). As the disease progresses, diffuse inflammatory T cell and B cell infiltrates, glia activation, diffuse myelin reduction and axonal injury become evident, which results in a more pronounced atrophy of the grey and white matter (Popescu and Lucchinetti 2012). Although the T cell composition of infiltrates does not differ as the disease develops, the relative proportion of B cells and plasma cells increases (Frischer et al. 2009). Microglia and macrophages remain in a chronic state of activation throughout the disease (Fischer et al. 2012). Eventually the inflammation becomes organized inside the CNS, with fewer invading cells observed in the lesions during progression. Indeed, in SPMS have been reported tertiary lymphoid structures – B-cell follicle-like structures – in the meninges, suggesting that B-cell maturation can be sustained locally within the CNS and therefore contribute to a compartmentalized humoral immune response (Magliozzi et al. 2007, Howell et al. 2011). The presence of meningeal follicles in SPMS patients correlates with an accelerated clinical disease (earlier age of clinical onset, faster disease progression and earlier age of death) perhaps due to a more pronounced demyelination, microglia activation and loss of neurites in the cortex. Notably, these meningeal B-cell follicles were found adjacent to large subpial cortical lesions, suggesting that soluble factors diffusing from these structures may have a pathogenic role by exacerbating

the detrimental effects of humoral immunity (Magliozzi et al. 2007). In addition, transcriptome analysis of clonally expanded B cells in cerebrospinal fluid (CSF) has shown that they are responsible for oligoclonal bands production (Obermeier et al. 2008, Obermeier et al. 2011). Also, B cells in MS lesions show somatic hypermutation, implying antigen-driven expansion (Qin et al. 1998). These data support that B-cell follicle-like structures may contribute to cortical demyelination and tissue injury at later stages.

2.2.5. Therapeutic strategies in multiple sclerosis

Although there is no cure for MS, in recent years significant advances have been made towards the treatment of this disease, aiming to obtain more effective and safer options. Pharmacological therapy of MS includes symptomatic drugs, treatment for relapses (corticosteroid and intravenous immunoglobulin) and disease modifying therapies (DMTs) that have an impact on relapse rate, disability accumulation and radiological outcomes (Fenu et al. 2015). These DMTs can be further classified as immunomodulators or immunosuppressors, depending on their ability to modulate or suppress immune response.

Several therapeutic DMTs approved by the Authority Agencies are able to reduce disease activity and progression in patients with relapsing forms of MS. According to their level of efficacy and risk of side effects, DMTs can be classified as lower-risk drugs having a good safety profile but a lower degree of clinical and neuroradiological efficacy (interferon beta-1, glatiramer acetate and teriflunimide), moderate-risk drugs [dimethyl fumarate (DMF), fingolimod, daclizumab and ocrelizumab], and higher-risk drugs associated with possible severe adverse events compensated by a very high anti-inflammatory activity (mitoxantrone, alemtuzumab, cladribine and natalizumab) (Comi et al. 2017, Dendrou and Fugger 2017). Besides, currently two different approaches can be used in MS therapy: 1) a chronic (or maintenance or escalation) therapy that is continuously administered and actively optimized according to disease activity; 2) an induction therapy, which involves pulsed immune reconstitution that is capable of transiently or permanently induce immune reset, producing long-lasting drug-free remission. Induction therapy can be followed by no treatment, treatment as needed, or maintenance therapy with another drug with lower efficacy but better safety (Coles 2015, Giovannoni et al. 2015, Wiendl 2017).

Concerning DMF, this compound was approved in 2013 as MS therapy by FDA and EMA (Wingerchuk and Weinshenker 2016). This drug, following oral administration is rapidly hydrolyzed to the bioactive metabolite monomethyl fumarate. DMF was described to significantly reduce the relapse rate by 53% and magnetic resonance imaging activity (accumulation of gadolinium-enhancing lesions or new or enlarging T2 lesions) by 90% (Fox 2012, Fox et al. 2012, Goldenberg 2012). Further, a 38% reduction in disability progression has been reported in one phase III DMF clinical trial (Gold et al. 2012). The mechanism of action of DMF in MS is not completely understood, but is thought to have a dual effect as anti-oxidant and anti-inflammatory agent. The antioxidant effect is associated with the activation of the transcription factor nuclear factor erythroid 2-related factor 2 (Nrf2) and increased glutathione production, protecting cells against oxidative stress. Curiously, it was demonstrated that DMF-mediated cytoprotection occurs

via Nrf2, oxidative stress induced growth inhibitor 1, and p53, as described following monomethyl fumarate administration to human spinal cord astrocytes (Brennan et al. 2016, Brennan et al. 2017). DMF also inhibits the transcription factor NF-κB, reducing the release of inflammatory cytokines (Phillips and Fox 2013). In addition, its anti-inflammatory properties have also been linked to its ability to modulate dendritic cell activation, Th cell differentiation, and regulatory B cell induction (Salmen and Gold 2014).

Some of these therapies are showing a high level of efficacy, with an acceptable adverse effect profile. Nevertheless, present therapies have significant limitations in slowing disease progression, reverse disability and are sometimes associated with significant side effects, therefore identifying more effective treatments with a broader efficacy is an important goal for clinical research in MS.

3. S100B protein

S100B was the first S100 protein to be identified and isolated from bovine brain extracts, as homodimer and heterodimer (Moore, 1965; Moore et al., 1968; (Cocchia and Michetti 1981). It was originally discovered in glial cells, being in fact abundantly expressed by these type of cells from both the CNS and peripheral nervous system (Donato, 2003; Rambotti et al., 1989), and makes up approximately 0.5% of all cerebral proteins (Sorci et al. 1998). Within the CNS, S100B is mainly expressed by astrocytes and some OL of hippocampus and brain cortex, although low levels are also present in certain neuronal subpopulations and microglia (Richter-Landsberg and Heinrich 1995, Hachem et al. 2005, Gerlach et al. 2006, Steiner et al. 2007). Outside the CNS, S100B is expressed at a variable abundance in a restrict number of cell types from different organs and tissues, such as kidney epithelial cells, pituicytes, ependymocytes, chondrocytes, adipocytes (Michetti et al. 1983), ependymal cells, enteric glial cells, retinal Muller cells (Ludwin et al. 1976, Brockes et al. 1979, Ferri et al. 1982, Didier et al. 1986), melanocytes, Langerhans cells, dendritic cells, certain lymphocyte subpopulations, skeletal myofibers, myoblasts and muscle satellite cells (Cocchia et al. 1983, Michetti et al. 1985, Tubaro et al. 2011). Moreover, the majority of S100B is located diffusely in the cytoplasm in a soluble form. However around 7% binds to intracellular membranes, centrosomes, MT and type III intermediate filaments. (Donato, 2003, Donato 2007, Donato et al. 2009, Sorci et al. 2010).

S100B levels are positively associated with age, fluctuating throughout development, and highly conditioned by external stimulating factors. S100B expression is first detected in the brain at E14 and at birth it is found in high levels, which slightly decrease thereafter. Notwithstanding, these levels increase with growth and development of CNS until adulthood, when S100B levels stabilize. Astrocytes present an average concentration of 10 μM of the S100B protein in physiological states (Sorci et al. 2010), being part released constitutively (Shashoua et al. 1984, Van Eldik and Zimmer 1987), or increasingly secreted upon stimulation by several agents (Ciccarelli et al. 1999, Edwards and Robinson 2006). In fact, S100B secretion is enhanced upon exposure to TNF-α, IL-1β, serotonin, lysophosphatidic acid, high levels of cytosolic Ca^{2+}, low levels of glutamate, metabolic stress (Edwards and Robinson 2006) and serum deprivation

(Steiner et al. 2008). In contrast, decreased S100B expression may be triggered by external factors that may involve high levels of glucose, glutamate and IFN-γ (Ciccarelli et al. 1999, Halonen et al. 2006), inhibition of Src kinase activity, cell confluence, Ca^{2+} channel blockade and gap junction inhibition (Sorci et al., 2010).

Concerning the presence of S100B protein in extracellular biological fluids, it was detected for the first time in the CSF of MS patients (Michetti et al. 1979). Since then, research of S100B has been extended to other biological fluids including peripheral blood (Kato et al. 1983), cord blood (Gazzolo et al. 2000), amniotic fluid (Gazzolo et al. 2001), urine (Gazzolo et al. 2005) and saliva (Gazzolo et al. 2005). It is noteworthy that S100B is also present in human milk and at greater quantities than those detected in CSF, blood or urine (Gazzolo et al. 2003, Gazzolo et al. 2005, Galvano et al. 2009). Besides the biological fluids, S100B can also be detected in brain tissue, having been found primarily in brains from patients with Alzheimer's disease (Griffin et al. 1989).

3.1. Cellular roles

The involvement of S100B in cellular events (Figure I.7.) has been extensively studied and includes the regulation of several critical processes including cell proliferation and differentiation; the control of the assembly of cytoskeleton components; cell morphology and locomotion; intracellular Ca^{2+} homeostasis; protein phosphorylation and degradation; transcription; enzyme activity and metabolism (Donato et al. 2009, Sorci et al. 2010). Besides being implicated in the regulation of intracellular activities, S100B can also exhibit cytokine-like activity being secreted, thereby affecting cellular activities in a paracrine, autocrine and endocrine manner (Sorci et al. 2010).

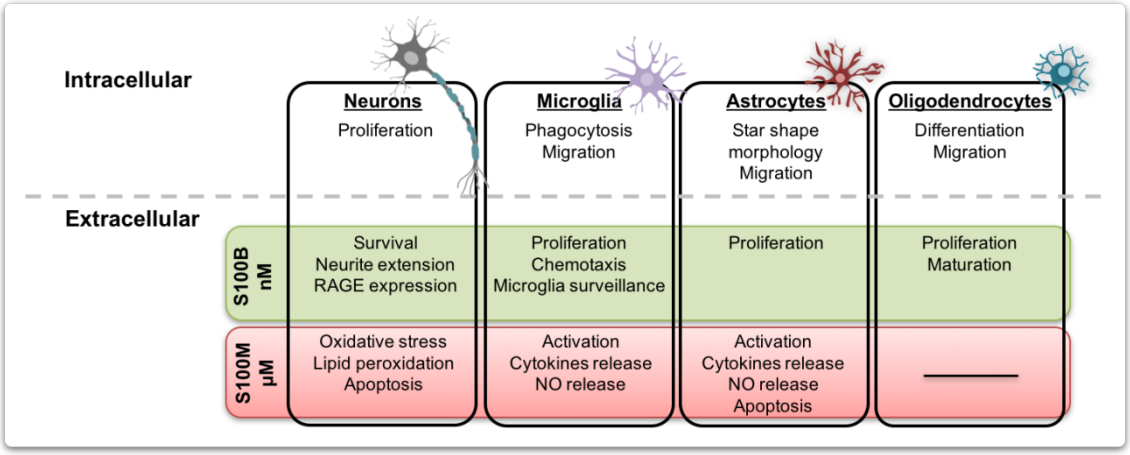

Figure I.7. Schematic representation of S100B regulatory effects. S100B can have both (**A**) intracellular and (**B**) extracellular roles. The extracellular effects that S100B exerts on different cells depend of the concentration attained, being beneficial in the range of nM or detrimental when reaching µM levels.

Additionally, S100B monomers have the ability to assemble into more complex oligomers, such as tetramers, hexamers and octamers. Therefore, the distinct functions of this protein might rely on both the degree of oligomerization and the type of bond that holds the monomers (Donato, 2007). Indeed, tetrameric forms of S100B induced stronger activation of cell growth and promoted cell survival (Ostendorp et al. 2007), whereas dimeric forms of S100B are mainly associated with

intracellular roles, such as modulation of MT assembly and regulation of cell cycle by interaction with transcription factors in a Ca^{2+}-dependent manner (Davey et al., 2001). Such cellular functions require specific binding to cell receptors such as the receptor for advanced glycation end products (RAGE), to which S100B binds preferentially when in tetrameric/octameric forms promoting RAGE dimerization (Ostendorp et al., 2007).

3.1.1. Intracellular roles

Within cells S100B might act as an intracellular regulator in a Ca^{2+}-dependent manner. Indeed, upon Ca^{2+} binding S100B dimmers suffer a conformational change exposing the hydrophobic residues, and consequently creating a hydrophobic cleft through which S100B can bind to target proteins (Iuvone et al., 2007). Many target proteins of S100B have been identified involving S100B in diverse intracellular events as regulation of Ca^{2+} homeostasis, cell proliferation and differentiation, protein phosphorylation and degradation, transcription, enzyme and channel activities, assembly of cytoskeleton components, cell morphology and locomotion, dark adaptation of photoreceptors and the innate inflammatory response (Donato et al. 2009, Sorci et al. 2010).

Within the CNS, S100B has been shown to play an important role in the regulation of neuronal (Arcuri et al. 2005) and astroglial (Brozzi et al. 2009) proliferation and differentiation during early development stages through PI3K activation and respective downstream signalling pathways, by enhancing cell proliferation and reducing cell differentiation. In fact, it was shown that S100B influences the cell division pathway by binding and activating the nuclear Dbf2, which is implicated in the regulation of cell division and morphology (Tamaskovic et al. 2003). In addition, S100B inhibits tumor suppressor p53 phosphorylation and activation, which increases cell proliferation (Markowitz et al. 2004).

S100B interacts with cytoskeleton components, modulating MT assembly and disassembly in a Ca^{2+}- and pH-dependent manner (Richter-Landsberg and Heinrich 1995, Garbuglia et al. 1998, Sorci et al. 1998). Interestingly, disassembly of MTs results in enhanced S100B release (Davey et al. 2001). In addition, S100B also inhibits the assembly of type III intermediate filaments, such as glial fibrillary acidic protein (GFAP) and desmin (Garbuglia et al. 1998, Sorci et al. 1998). Besides, it is suggested that S100B is involved in actomyosin stabilization since it interacts with caldesmon inhibiting its phosphorylation by cdc2 kinase and ERK1/2 MAPK (Hai and Gu 2006) and consequently the actomyosin ATPase activity (Skripnikova and Gusev 1989). S100B is also involved in astrocytic improved migratory ability and star-shaped morphology favouring cell locomotion by promoting the formation of stress fibers and of cytoplasmic extensions, in a PI3-K dependent manner (Brozzi et al., 2009; Watanabe et al., 2004). Additionally, S100B was found to be associated with microglia microtubule-like structures and centrosomes, suggesting its involvement in the phagocytic ability of these glial cells (Adami et al., 2001).

3.1.2. Extracellular roles

As mentioned above, S100B is primarily and constitutively secreted by astrocytes within CNS, which is believed to occur through activation of metabotropic glutamate receptor 3 in a neural and synaptic activity dependent manner (Sakatani et al., 2008; Shashoua et al., 1984). Once in the extracellular space S100B can exert both neuroprotective and toxic effects on neurons, microglia and astrocytes depending on the concentration (Van Eldik and Wainwright, 2003). Moreover, several evidences suggest that these extracellular S100B effects are mainly mediated by the binding to RAGE, and their interaction is somehow responsible for the protein dual roles (Ostendorp et al. 2007) Sorci et al., 2013). Additionally, S100B-RAGE mediated roles might depend on the intensity and extent of activation of the receptor, as well as, on its level of expression in CNS cells (Donato, 2007; Leclerc et al., 2009).

When present extracellularly at low and physiological concentrations (nM), S100B behaves as a signalling trophic protein, stimulating cell proliferation and migration, and inhibiting apoptosis and cell differentiation (Sorci et al. 2010). In neurons, S100B exert trophic effects by stimulating neurite outgrowth and regeneration, synaptogenesis and long-term plasticity, neurogenesis and survival during development and following injury (such as stress and Aβ toxicity). These effects occur via stimulation of ERK1/2 and NF-κB, as well as, upregulation of the anti-apoptotic protein Bcl-2 (Barger et al. 1995, Huttunen et al. 2000, Arcuri et al. 2005, Businaro et al. 2006). Concerning glial cells, S100B prevents astrocytic (Brozzi et al. 2009) and microglial activation via STAT3 pathway (Zhang et al., 2011b) and consequent inflammation (Zhang et al. 2011). Additionally, stimulates astrocytic proliferation by phosphorylation of ERK1/2 and glutamate uptake, and enhances microglial chemotactic ability (Gonçalves et al., 2000; Reali et al., 2005; Selinfreund et al., 1991; Zhang et al., 2011b).

In contrast, at higher concentrations (μM), S100B plays a main role in neurodegeneration and on the exacerbation of the inflammatory response, by triggering glial activation with the expression and release of proinflammatory cytokines and inflammatory stress-related enzymes leading to cell dysfunction and death (Bianchi et al. 2010, Sorci et al. 2010, Astrand et al. 2013). Indeed, excessive extracellular levels of S100B have been shown to induce neuronal dysfunction or apoptosis in a direct action (Mariggio et al. 1994), or as a result of gliosis with the release of nitric oxide (NO) and proinflammatory cytokines (Hu et al. 1997, Koppal et al. 2001). The direct effect of S100B is related with the activation of the pro-apoptotic Ras/MEK/ERK pathway and subsequent overproduction of reactive oxygen species (ROS) and NO (Businaro et al., 2006; Huttunen et al., 2000), as well as, with enhanced Aβ toxicity (Sorci et al. 2010, Astrand et al. 2013). Again these extracellular toxic S100B effects are mainly attributed to interaction with RAGE (Ostendorp et al. 2007). S100B promotes gliosis in a RAGE-dependent manner via RAGE/Rac-1-Cdc42 pathway (Villarreal et al. 2014), leading to the release of inflammatory cytokines (Bianchi et al. 2007, Brozzi et al. 2009) in response to activation of NF-κB (Bianchi et al. 2010). More specifically, in astrocytes, S100B overstimulates inducible NO synthase (iNOS) activity (Lam et al., 2001; Petrova et al., 2000) and promotes IL-1β, IL-6 and TNF-α release (Hu and Van Eldik, 1999), whereas in microglia acts synergistically with cofactors like bacterial endotoxin or IFN-γ, upregulating iNOS expression and NO release (Adami et al., 2001; Petrova et al., 2000).

3.1.3. Role in oligodendrogenesis

Data regarding oligodendrial cells revealed the presence of an expression pattern of S100B along their differentiation and maturation (Deloulme et al., 2004), nevertheless its role in oligodendrogenesis is yet not fully understood.

In developing mouse brain and along adulthood, S100B is mostly expressed during the intermediate cell stages in the nuclei of OPC committed to differentiate into mature OL (Deloulme et al. 2004). Also, S100B expression was shown to greatly increase during the transition between non-myelinating preOL and mature myelinating OL of mouse embryonic hindbrain and SC, being the S100B expression in these populations comparable to that of astrocytes (Deloulme et al. 2004). This elevated expression might be explained by the association of S100B with cytoskeleton components, which in turn are highly present in the extensions and membranous sheaths of mature OL (Deloulme et al., 2004; Zhang, 2001). These findings implicate S100B in OPC proliferation and differentiation into mature OL. In fact, S100B absence induced no effects in the number of OPC, but delayed morphological transformation and maturation of preOL cells *in vitro.* On the other hand*,* mice lacking S100B showed impaired OPC maturation upon a demyelinating insult (Deloulme et al. 2004). In addition, the increased S100B expression during the transition from non-myelinating to myelinating cells, when elaboration of myelin sheaths occurs concomitantly with a drastic loss of arborisation, suggests that S100B could modulate OL shape, as already demonstrated for other CNS cells. Accordingly, S100B is associated with microtubular structures in cultured OL, such as microtubule-associated protein 2 and tau (LoPresti 2002), whose phosphorylation by protein kinase II is inhibited by S100B (Baudier and Cole 1988). Moreover, mitochondrial ATAD3A protein, involved in the regulation of mitochondrial dynamics and diverse cell responses, has been also identified as a target of S100B in OPC (Gilquin et al. 2010). Since OPC differentiation requires *de novo* mitochondrial protein synthesis, this study suggests that ATAD3A is required for a proper cell growth and differentiation.

Regarding extracellular S100B, mature OL from the OL-93 cell line secrete S100B at a higher level than astrocytes under serum and glucose deprivation conditions (Steiner et al., 2008a), but it was not reported any benefit and/or deleterious effect in their development. Nevertheless, elevated levels of S100B can indirectly lead to OL injury and/or death due to their increased susceptibility to S100B-induced oxidative damage and mitochondrial injury, as well as excitotoxicity and inflammatory cytokines, which in turn might be detrimental for OL proper development and function.

3.1.4. S100B as a biomarker of brain damage

S100B physiological concentration is necessary for development, plasticity, and damage repair (Zimmer et al. 1995). Nonetheless, increased S100B concentrations have been detected in several neurodegenerative and inflammatory diseases. Also, cellular and tissue distribution of S100B is altered in numerous neuropathological conditions, including those induced by environmental stress, infection, trauma and stroke (Sorci et al. 2010). Therefore, both intra- and

extracellular mRNA and protein levels of this protein have been used as markers of glia damage, such as compromised/dying astro- or oligodendroglial cells with ability to activate the response of immune cells, in a variety of brain injury conditions (Marks et al. 1996, Rothermundt et al. 2003, Marenholz et al. 2004, Kleindienst and Ross Bullock 2006, Goncalves et al. 2008, Yardan et al. 2011). Indeed, recent studies correlate S100B variations with neuroimaging alterations, intracerebral pressure measurements and neurological signs (Bloomfield et al. 2007). Although in many instances its release may be an effect of the condition rather the cause, experimental findings point to S100B as having a possible role as a biomarker of CNS injury and prognosis, as well as having an important therapeutic potential.

3.1.5. S100B in peri- and neonatal injury

S100B has already been identified as a potential biomarker in cases of premature and traumatic brain injury, once it has been reported to be augmented in the CSF and serum of patients following such traumas (Beharier et al., 2013; Kleindienst and Ross Bullock, 2006). Indeed, higher levels of S100B in the urine have been described more in premature infants, than in term newborns (Gazzolo et al. 2007). Furthermore, elevated S100B concentrations were also found in the urine, blood, serum or CSF of a variety of neonatal brain injuries or diseases involving myelin sheath damage, such as hypoxic-ischemic encephalopathy (Gazzolo et al. 2004), IVH (Gazzolo et al. 1999, Gazzolo et al. 2001), PVL and WMI (Huang et al. 2015, Zhou et al. 2015). Besides, S100B can be also used to monitor the impact of prenatal drug exposure (Michetti et al. 2012). To note, S100B levels have been correlated with the extent of brain lesions and with worse long-term prognosis in preterm infants affected by perinatal hypoxia (Blennow et al. 2001), post-hemorrhagic ventricular dilation (Whitelaw et al. 2001) and IVH (Gazzolo et al. 2001). Excessive S100B concentrations are also associated with a poorer prognosis in premature infants with PVL (Huang et al. 2015) and to sustained brain injury of preterm infants with ischemic brain damage (Chiang et al. 2015). In addition, S100B was indicated as a potential biomarker of brain injury and clinically unfavourable outcomes in children with CNS infections (Peng et al. 2017). So, it is crucial to understand the pathogenesis associated with excessive S100B levels during neurodevelopment.

3.1.6. S100B in multiple sclerosis

The role of S100B in MS has been greatly debatable. Initial data reported increased S100B concentrations in the CSF of patients with MS, especially in the acute phase of exacerbation of the disease, being almost undetectable during remission (Michetti et al. 1979). Accordingly, subsequent studies revealed a maximal S100B concentration at CSF and serum following onset of an acute exacerbation, which decreased thereafter (Massaro et al. 1985, Missler et al. 1997). Later it was described a trend of increasing S100B levels in CSF and serum from PPMS to SPMS to RRMS, whereas the highest S100B levels were observed in the inflammatory course of RRMS. Notwithstanding, these levels significantly decreased after therapy with an immunosuppressive agent (Bartosik-Psujek et al., 2011; Petzold et al., 2002). In accordance, our group recently

showed a significant increase of S100B concentrations in the CSF and serum of RRMS patients at the time of diagnosis (Barateiro et al. 2016).

Studies using *post-mortem* brain tissue of MS patients showed elevated S100B levels in the white matter (subacute plaques) and in grey matter (cortical zone) in the RRMS, allowing to distinguish between this and the progressive phases (Petzold et al. 2002). In agreement, our recent data demonstrated that S100B is overexpressed both in active and chronic active lesions mainly by astrocytes. While in active lesions S100B surrounds the demyelinated area, in chronic active ones S100B is diffusely expressed within the demyelinated areas. RAGE was also highly expressed by macrophages/microglia in active MS lesions (Barateiro et al. 2016). Similarly, it was described increased RAGE immunostaining in the hippocampus (Sternberg et al. 2011) and SC of MS patients, particularly evident in mononuclear phagocytes and $CD4^+$T cells although its expression was also observed in neurons, vasculature and inflammatory cells (Yan et al. 2003).

In addition, using an *ex vivo* model we demonstrated that S100B is overexpressed and mainly released by astrocytes following LPC-induced demyelination, in parallel with boosted glia reactivity, enhanced gene expression of proinflammatory cytokines and inflammasome-related molecules (Barateiro et al. 2016). Interestingly, S100B blockade attenuated demyelination that was accompanied by a decrease of glia activation and expression of proinflammatory cytokines.

These evidences suggest that S100B might have a role in the pathogenesis of MS. Therefore, S100B might be not only a potential target but also a biomarker for MS diagnosis and prognosis allowing the stratification of different MS stages.

3.1.7. S100B modulators

Taking in account all the evidences relying on the increased S100B expression in myelin-related diseases and its toxic effects, it can be assumed that its inhibition may have a beneficial outcome. In fact, S100B blockade with pentamidine, arundic acid, anti-S100B antibodies and specific inhibitor (TRTK-12) has already been tested in other disease models with promising results.

In addition to the antiprotozoal activity, pentamidine was also one of the first compounds found to inhibit S100B-mediated effects due to its ability to inhibit S100B/p53 interaction (Markowitz et al. 2004). Pentamidine significantly decreases C6 rat glioma cell proliferation and increases the BAX/Bcl-2 ratio, demonstrating its pro-apoptotic role, while reducing cell migration by inhibiting aquaporin 4 and matrix metalloproteinase-2 (Capoccia et al. 2015). In *in vivo* studies, pentamidine reduces S100B and its receptor RAGE expression, induces p53 expression in astrocytes and reduces the expression of proinflammatory mediators and markers, such as GFAP, iNOS and cyclooxygenase-2 (COX-2) (Cirillo et al. 2015). Additionally, pentamidine significantly inhibits S100B-induced lipid peroxidation, p-p38 MAPK phosphorylation and, consequently, NF-κB activation (Esposito et al. 2012, Cirillo et al. 2015). More importantly, pentamidine is also able to control macrophages infiltration (Esposito et al. 2012).

Arundic acid [(R)-(-)-2-propyloctanoic acid, ONO-2506] inhibits S100B synthesis, reducing its the levels of mRNA and protein in astrocytes (Tateishi et al. 2002, Asano et al. 2005). Interestingly, in the CNS arundic acid acts exclusively on astrocytes, modulating and preventing their excessive activation that may be harmful. In *in vitro* and *in vivo* studies, arundic acid leads to a decrease of the number of apoptotic cells, astrocyte reactivity, NGF secretion and mRNA expression of iNOS and COX-2. Conversely, it enhanced the expression of glutamate transporters (glutamate transporter subtype 1 and glutamate/aspartate transporter), and of $GABA_A$ receptors, in a dose-dependent manner without affecting GFAP expression (Mori et al. 2004, Asano et al. 2005). In a mouse model of Alzheimer's disease, the Tg2576, S100B was shown to drive brain inflammation and gliosis that accelerated cerebral amyloidosis, which was suppressed by arundic acid through the attenuation of S100B biosynthesis (Mori 2006).

Anti-S100B neutralizing antibodies have been consistently used to reverse elevated S100B levels and increased S100B expression in astrocytes, as well as, to prevent its effects on impaired glutamate uptake (Tramontina et al. 2006) and long-term potentiation (O'Dowd et al. 1997, Rebaudo et al. 2000, Motin et al. 2002). More recently, anti-S100B was shown to reduce LPC-induced demyelination, astrocyte reactivity and the expression of inflammatory and inflammasome-related molecules (Barateiro et al. 2016).

The TRTK-12, which binds to the p53-binding site on S100B, was shown to block cAMP-induced astrocyte stellation in a concentration-dependent manner (Frizzo et al. 2004) and GFAP phosphorylation (Frizzo et al. 2004). In additional studies, TRTK-12 also reduced abnormal dendritic growth and morphology of Purkinje cells in slice cultures and improved behavioural impairment in an animal model of spinocerebellar ataxia (Vig et al. 2011).

3.1.8. RAGE modulators

RAGE-ligand interaction may be blocked by different strategies including: competitor soluble RAGE isoform (sRAGE), specific introduction of a dominant-negative form of RAGE (DN-RAGE), or the specific inhibitor N-benzyl-4-chloro-N-cyclohe-xylbenzamide (FPS-ZM1) (Wautier et al. 2016).

sRAGE may act as a competitive inhibitor of RAGE, neutralizing the ligand-RAGE interaction and consequently blocking the signal transduction pathway that ultimately leads to cellular damage (Santilli et al. 2009, Koyama et al. 2014). Several studies reported that sRAGE successfully prevents or reverses RAGE-signalling effects in several animal models of RAGE-mediated disorders, such as diabetic and non-diabetic atherothrombosis, ischemia/reperfusion injury, collagen-induced arthritis, colitis, tumor cell migration and invasion, and neurodegeneration (Santilli et al. 2009, Vazzana et al. 2009). In fact, sRAGE prevented RAGE-mediated Aβ toxicity (Deane et al. 2004) delaying the development and progression of the inflammatory progress and cellular stress in Alzheimer's disease models (Yamagishi and Matsui 2010). It also delayed amyotrophic lateral sclerosis progression in SOD1 mice (Juranek et al. 2016), and suppressed EAE by selective blockade of CD4+ T cells and markedly decreased infiltration of CNS by immune and inflammatory cells (Yan et al. 2003).

Notably, the truncated DN-RAGE form in *in vivo* models had also a marked effect on RAGE-mediated toxicity. Introduction of DN-RAGE into an animal model of Alzheimer's disease, the transgenic mAPP mice, significantly counteracted the long term potentiation dysfunction (Yan et al. 1996, Origlia et al. 2009), improved learning/memory, and alleviated neuropathology (Yan et al. 1996) induced by Aβ in mAPP/DN-RAGE mice. Also in double transgenic mice overexpressing both mAPP/RAGE, the DN-RAGE form was able to preserve the spatial learning/memory and diminished the neuropathological alterations (Arancio et al. 2004). In addition, mAPP/DN-RAGE mice also exhibited an enhancement of Aβ-mediated enhancement of p38 MAPK phosphorylation in cortical neurons (Origlia et al. 2008), and p38 MAPK and ERK1/2 in microglia (Fang et al. 2010). Moreover, in microglia of mAPP/DN-RAGE mice the release of cytokine was delayed and attenuated. More importantly, mAPP/DN-RAGE mice presented no significant microglial and astrocyte infiltration in the cerebral cortex and hippocampus (Yan et al. 2012).

FPS-ZM1, a high-affinity but nontoxic RAGE-specific inhibitor, was found to inhibit β-secretase activity, Aβ (Aβ40 and Aβ42) production, and microglia activation (Deane et al. 2012, Hong et al. 2016, Chen et al. 2017, Shen et al. 2017). In a rat model of Parkinson's disease, the 6-OHDA, FPS-ZM1 inhibited RAGE expression, the activation of ERK1/2, Src and NF-κB. RAGE inhibition also reduced the levels of gliosis markers and glial activation, as well as, proinflammatory cytokines. Moreover, FPS-ZM1 attenuated 6-OHDA-induced dopaminergic denervation, locomotor and exploratory deficits (Gasparotto et al. 2017). Concerning a more specific role of FPS-ZM1 in microglial cells, it was demonstrated that this antagonist significantly reduces AGEs-mediated activation of BV2 cells by inhibiting ROS induction, downregulating iNOS, COX-2, NLRP3 and nuclear translocation of NF-κB. In addition, FPS-ZM1 led to a decreased M1 and increased M2 phenotype in BV2 cells (Chen et al. 2017). Also in primary microglial cultures, FPS-ZM1 significantly suppressed AGEs-induced RAGE overexpression, RAGE-dependent microglial activation, nuclear translocation of NF-κB and the subsequent expression of downstream inflammatory mediators (TNF-α, IL-1β, COX-2, prostaglandin E2, iNOS and NO). In contrast, FPS-ZM1 elevated the levels of Nrf2 and heme oxygenase-1, that can protect cells from ROS (Shen et al. 2017). In a rat model of intracerebral hemorrhage, FPS-ZM1 administration significantly improved blood–brain barrier damage, brain edema, motor dysfunction, and nerve fiber injury. These effects rely on the reduction of the expression of RAGE, NF-κB, proinflammatory mediators (IL-1β, IL-6, IL-8R, COX-2, iNOS) and matrix metallopeptidase-9. Moreover, FPS-ZM1 partially recovered the expression of decreased claudin-5 and occludin (Yang et al. 2015). Other study showed that FPS-ZM1 was able to inhibit primary tumor growth, angiogenesis, inflammatory cell recruitment and metastasis of breast cancer cells to the lung and liver (Kwak et al. 2017).

Overall, blockade of S100B-RAGE axis has been reported to play a neuroprotective role in several disorders associated with the CNS. Given the known involvement of S100B on myelin-associated disorders we may hypothesize that this effect may occur through RAGE engagement. Most attractive we may also conjecture that modulation of S100B-RAGE axis may be a potential

therapeutic strategy for prevention of damage and/or recovery improvement in inflammatory disorders involving white matter damage.

4. Global aims of the thesis

The main goal of the present work is to understand the **involvement of the S100B-RAGE axis in inflammatory-associated myelin damage, either during neurodevelopmental white matter damage, or upon demyelinating disorders**.

Specific aims are to:

1) **Evaluate whether excessive S100B levels can affect oligodendrogenesis, either during proliferation or initial differentiation, and whether it occurs through RAGE engagement.** For this we will treat primary cultures of OL with different concentrations of S100B, mimicking a physio- and a pathological situation, and evaluate OL differentiation. Next, we will co-treat primary OL with elevated levels of S100B in the presence of RAGE antagonist FPS-ZM1 and assay its protective role on oligodendrogenesis alterations. Then we will treat developmental OCSC obtained from wild-type Wistar rats with high S100B levels, in the absence or presence of RAGE antagonist FPS-ZM1, and evaluate deficits in myelin formation and inflammatory events in a tissue context.

2) **Assay if blockade of S100B-RAGE axis, using a specific RAGE antagonist, may prevent the pathogenesis observed following demyelination.** For this we will induce demyelination in OCSC obtained from wild-type Wistar rats using LPC, in the absence or presence of RAGE antagonist FPS-ZM1, and evaluate demyelination, neuronal damage and inflammatory events.

3) **Characterize the S100B-RAGE axis in the inflammatory EAE model and its modulation by the clinical drug DMF.** For this we will use, in collaboration with JJ Cerqueira, ICVS/i3B´s, Universidade do Minho, a cohort of animals that were divided in 3 groups: non-induced, EAE-induced, and EAE-induced and treated with DMF at clinical score peak. We will assay the expression of S100B and RAGE, as well as demyelination, neuronal integrity and inflammatory markers.

All together, we expect that the present study clarifies the role of S100B-RAGE axis in oligodendrogenesis and myelinogenesis during brain development and/or after injury, in order to define targets to be explored in the assessment of innovative neuroprotective strategies for inflammatory-related demyelinating diseases.

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Chapter II

Impaired oligodendrogenesis and myelination by elevated S100B levels during neurodevelopment

Gisela Santos[a], Andreia Barateiro[a], Cláudio M. Gomes[b], Dora Brites[a, c], Adelaide Fernandes[a, c']

[a]Neuron Glia Biology in Health and Disease, Research Institute for Medicines (iMed.ULisboa), Faculty of Pharmacy, Universidade de Lisboa, Lisboa, Portugal

[b]Faculdade de Ciências Universidade de Lisboa, Biosystems and Integrative Sciences Institute and Department of Chemistry and Biochemistry, Universidade de Lisboa, Campo Grande, Lisboa, Portugal

[c]Department of Biochemistry and Human Biology, Faculty of Pharmacy, Universidade de Lisboa, Lisboa, Portugal

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Abstract

High levels of the inflammatory molecule S100B protein have been identified in sera from several perinatal inflammatory conditions involving myelin damage and associated with an adverse prognosis or the emergence of sequelea. S100B is essential for oligodendrocyte (OL) differentiation and maturation, but it remains to be established if excessive levels of released S100B upon early brain injury are deleterious in the neurodevelopmental period. Here, we investigated this possibility by evaluating how elevated S100B affects oligodendrogenesis during this period. First, using primary cultures of OL we observed that damage-induced micromolar levels of S100B impair OL differentiation process. S100B elevated concentrations reduced both transition from immature $NG2^+$ oligodendrocyte precursor cells (OPC) to mature MBP^+ OL, and morphological maturation of differentiated OL. Interestingly, these effects were abolished by the use of receptor for advanced glycation end-products (RAGE) antagonist FPS-ZM1, suggesting an involvement of the S100B-RAGE axis on oligodendrogenesis impairment. Next, we used organotypic cerebellar slice cultures to explore the role of S100B in a more complex multicellular environment. Also in this model excessive S100B levels impaired oligodendrogenesis resulting in a reduced myelination. Further, elevated S100B levels compromised neuronal and synaptic integrity, while inducing astrogliosis, nuclear factor (NF)-kB activation and inflammation. Again, the FPS-ZM1 co-treatment prevented S100B-induced damaging effects. Overall, our results indicate that persistently elevated S100B levels have deleterious effects during the neurodevelopmental period through RAGE-dependent processes. Thus, targeting high S100B levels and/or S100B-RAGE interaction may constitute good therapeutic strategies to reduce brain injury, including deficits in neuronal architecture, synaptogenesis and myelination associated with perinatal inflammatory conditions.

1. Introduction

In the brain, the formation of myelin sheath by oligodendrocytes (OL) occurs predominantly postnatally and is a key requisite for brain development and efficient function of the central nervous system (CNS). The myelin membrane that wraps the axon is of critical importance for neuronal survival and integrity, providing them protection and nutritional support (Mei et al., 2013), as well as for synaptogenesis by improving conduction of action potentials (Franklin and ffrench-Constant, 2008).

Through OL development cells display different stages of maturation [for review see (Barateiro and Fernandes, 2014)]. Initially, immature oligodendrocyte precursor cells (OPC) expressing platelet-derived growth factor receptor (PDGFR)α and the proteoglycan neural-glial antigen 2 (NG2) have few and short multipolar ramifications. Upon differentiation to pre-myelinating OL, cells present long ramified branches and extend their processes towards multiple axons initiating the expression of myelin proteins. Lately, once axo-glial contact is established, mature myelinating OL expressing myelin proteolipid protein (PLP) and myelin basic protein (MBP) form membranes that enwrap sheaths around the axons with subsequent compaction forming the myelin sheath. During this process, OL maturation is driven by multiple signals that regulate their migration, proliferation and differentiation. For example, the transcription factors Olig1 and Olig2 are responsible for modulating OPC differentiation (Balabanov and Popko, 2005; Ligon et al., 2006). The Olig1 signalling is essential for the transcription of myelin proteins; so, its absence leads to OL arrest at an early stage of differentiation (Xin et al., 2005). Instead, Olig2 is required for specification to the OL lineage (Copray et al., 2006).

The last weeks of gestation and the first postnatal months are crucial periods for white matter maturation, being this period highly vulnerable to any kind of insult. Several cellular and molecular mechanisms, as inflammatory factors, have been implicated in pre-myelinating OL injury and death (Back and Rosenberg, 2014), resulting in myelin defects. Impaired myelination during the perinatal period will consequently result in axonal dysfunction and abnormal electrical conduction, therefore contributing to brain dysfunction and long-term neurological impairments. Curiously, among the different mediators involved in the inflammatory milieu, the S100B protein has been lately described to be elevated in urine, blood, serum or cerebrospinal fluid (CSF) of a variety of neonatal brain injuries and diseases involving myelin sheath damage, such as hypoxic-ischemic encephalopathy (Gazzolo et al., 2004), intraventricular haemorrhage (Gazzolo et al., 1999, 2001), periventricular leukomalacia (PVL) and white matter injury (Huang et al., 2015; Zhou et al., 2015). To note, high S100B levels have been associated with a poorer prognosis in premature infants with PVL (Huang et al., 2015) and indicated as a potential biomarker of brain injury and clinically unfavorable outcomes in children with CNS infections (Peng et al., 2017). So, it is crucial to understand the pathogenesis associated with excessive S100B levels during neurodevelopment.

S100B is a small $Ca2^{+}$ binding protein, initially regarded as specific to the nervous system, but later also identified in other tissues (Cocchia and Michetti, 1981). In the CNS, S100B has both intracellular and extracellular roles, being primarily secreted by astrocytes (Shashoua et al., 1984; Van Eldik and Zimmer, 1987). Within cells, S100B is known to regulate the proliferation and

differentiation of neurons and glia (Donato et al., 2009; Sorci et al., 2010). In the extracellular space, S100B can have different roles in a concentration-dependent manner (Van Eldik and Wainwright, 2003). At physiological concentrations within the nM range, S100B can stimulate cell proliferation and migration, and inhibit apoptosis and differentiation (Sorci et al., 2010). In fact, S100B regulates neurite outgrowth, synaptogenesis and long-term plasticity (Arcuri et al., 2005), while preventing astrocyte (Brozzi et al., 2009) and microglia activation and consequent inflammation (Zhang et al., 2011b). On the other hand, upon injury the released S100B may attain high concentrations in the μM range, having a main role in neurodegeneration and on the exacerbation of the inflammatory response, by triggering glial activation with the release of proinflammatory cytokines and inflammatory stress-related enzymes culminating in cell dysfunction and death (Astrand et al., 2013; Bianchi et al., 2010; Sorci et al., 2010). Evidence suggests that these extracellular S100B effects are mainly mediated through interactions with the receptor for advanced glycation end-products (RAGE) (Ostendorp et al., 2007), promoting gliosis via RAGE/Rac-1-Cdc42 pathway and a proinflammatory response by nuclear factor kappa B (NF-κB) activation (Villarreal et al., 2014).

Little is however known about the role of S100B in myelination. S100B is expressed in the nucleus of OPC and along adulthood, being needed for *in vitro* morphological transformation and maturation of preOL cells, and *in vivo* OPC maturation following a demyelinating insult (Deloulme et al., 2004). Moreover, we recently showed that S100B is highly released upon demyelination of mature organotypic cerebellar slice cultures (OCSC), while its neutralization reduces challenge-associated myelin deficits and inflammation (Barateiro et al., 2016a), suggesting that S100B plays a role in oligodendrogenesis.

In the current study we hypothesized that excessive S100B levels could impair proper oligodendrogenesis delaying OL differentiation and neurodevelopmental myelination through RAGE engagement. To address this question we used primary cultures of OL to evaluate direct S100B effects on OPC differentiation into mature OL, as well as developmental OCSC to study neuron-glia interactions leading to myelination and to the involvement of S100B-related inflammation. To further confirm whether S100B effects were mediated by RAGE engagement, parallel studies were performed with the RAGE-specific antagonist FPS-ZM1.

2. Materials and Methods

2.1. Animals

Animal care followed the recommendations of the European Convention for the Protection of Vertebrate Animals Used for Experimental and Other Scientific Purposes (Council Directive 86/609/EEC) and National Law 1005/92 (rules for protection of experimental animals). All animal procedures were approved by the Institutional animal care and use committee. Every effort was made to minimize the number of animals used and their suffering.

2.2. *In vitro* model – primary oligodendrocyte cell culture and treatment

Primary OPC cultures were obtained from post-natal day (P)1 to P2 neonatal rat cortices as previously described by Chen (Chen et al., 2007), with minor modifications established by our group (Barateiro et al., 2012). Briefly, a mixed glial culture isolated from neonatal Wistar rats was grown in Dulbecco's modified Eagle's medium (DMEM) (Biochrom AG, Berlin, Germany) supplemented with 20% fetal bovine serum (FBS, Biochrom AG), 1 mM sodium pyruvate (Biochrom AG), 2 mM L-glutamine (Biochrom AG) and 0.5% antibiotic antimycotic (Sigma Chemical Co, St. Louis, MO, USA), with medium changes every 2 days until 10 days *in vitro* (DIV). To remove the microglia on the top of the mixed glial cultures, the flasks were shaken for 1 h at 260 r.p.m. on an orbital shaker, media changed and shaken at 260 r.p.m. overnight to dislodge the loosely attached OPC. Isolated OPC were further purified from contaminating microglia by a differential adhesion step. Pure OPC were plated in SATO media [L-glutamine (4 mM, Biochrom AG), putrescine (16 µg/mL, Sigma), L-thyroxine (400 µg/mL, Sigma), triiodo-thyronine (400 µg/mL, Sigma), progesterone (6.2 ng/mL, Sigma), sodium selenite (5 ng/mL, Sigma), bovine serum albumin (BSA) V (100 µg/mL, Sigma), insulin (5 µg/mL, Sigma), holo-transferrin (50 µg/mL, Sigma)] supplemented with 1% penicillin/streptomycin, 1% insulin-transferrin-sodium selenite (Sigma), 0.5% FBS, 10 ng/mL platelet-derived growth factor (PDGF)-AA (PeproTech, Rocky Hill, NJ) and 10 ng/mL fibroblast growth factor (FGF)-2 (PeproTech) in order to promote OPC adherence in a pro-survival and proliferative environment. As previously described (Barateiro et al., 2012), in these OPC cultures, prior to differentiation induction, more than 99% of the cells express OPC markers (NG2 and A2B5), with less than 1% of glial fibrillary acidic protein (GFAP) positive astrocytes and no calcium-binding adapter molecule 1 (Iba1) positive microglia. After 1 DIV, differentiation was induced by replacing PDGF-AA and FGF-2 by 3% FBS in SATO medium and cells were kept in culture for 7 DIV in order to obtain an enriched culture of mature OL, with more than 60% of MBP^+ cells.

To evaluate the effects of extracellular S100B, we produced highly pure recombinant S100B in *Escherichia coli*, which was subsequently purified to homogeneity using chromatographic methods and a previously established protocol as detailed in (Botelho et al., 2012). In these experiments, OPC were incubated for 24 h in the absence (control) or in the presence of S100B at 10 nM or 1 µM, to mimic low or pathology-related high levels, respectively. OPC were treated just after isolation (1 DIV), to assess S100B effect during the proliferation phase, or at 2 DIV, to test S100B effect in initial differentiation. Since the effects of high S100B were mainly observed during initial OPC differentiation, we used this time-point to assess the relevance of S100B-RAGE interaction in final outcomes. For that, OPC were incubated during the first 24 h of differentiation with S100B (1 µM) or S100B plus RAGE antagonist FPS- ZM1 (Calbiochem, La Jolla, CA) with a K_i = 230 nM for S100B-RAGE interaction inhibition (Deane et al., 2012). We used FPS-ZM1 in a concentration of 3 µM, more than 10x the K_i, to assure a maximal inhibition of S100B interaction with RAGE under non-toxic conditions. Following incubation the medium was replaced by basal differentiation medium until 7 DIV when cells were collected and stored in TRIzol® reagent

(Invitrogen, Carlsbad, CA, USA) at 20 °C for RNA extraction; or fixed in 4% paraformaldehyde (PFA) for immunohistochemistry assays.

2.3. *Ex vivo* model – organotypic rat cerebellar slice cultures and treatment

To study the role of S100B during neurodevelopment we used OCSC. This *ex vivo* model preserves the complex multicellular environment, maintaining cell relationships and the extracellular matrix in a relatively intact three-dimensional structure. Indeed, it was demonstrated that both OPC and mature OL are present in OCSC, and that compact myelin is formed (Zhang et al., 2011a), indicating its suitability to evaluate the OL maturation process and myelination, thus avoiding several sources of artifacts from other experimental systems (Ghoumari et al., 2003; Kasparov et al., 2002; Schnadelbach et al., 2001). Cerebella were isolated from P7 rat pup dissected brains and parasagittal slices of 400 µm obtained using a McIlwain tissue chopper. Four slices of different animals were placed into membrane culture inserts (BD Falcon, Lincoln Park, NJ, USA) in 6-well cell culture plates in an air-liquid interface at 37 °C in 5% CO_2 conditioned atmosphere, and kept in culture until 7 DIV to allow full myelination (Birgbauer et al., 2004). Culture media consisted of 50% minimal essential media (MEM, Gibco, Life Technologies, Inc., Grand Island, USA), 25% heat- inactivated horse serum (Gibco), 25% Earl's balanced salt solution (EBSS, Gibco), 6.5 mg/mL glucose, 25 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) (Biochrom AG), 1% L-gluta- mine (Biochrom AG) and 1% antibiotic-antimycotic (Sigma). Half media was replaced every day and at 3 DIV, slice culture media was totally changed to a serum-free media that consists of Neurobasal-A (Gibco) supplemented with 2% B-27 (Gibco), 2 mM L-glutamine, 36 mM glucose, 1% of antibiotic-antimycotic and 25 mM HEPES, in order to improve neuronal viability. Since we wanted to evaluate the effect of S100B in *de novo* myelination, cerebellar slices were kept in culture until 3 DIV to allow the clearance of debris and the stabilization of the system, and then incubated with S100B (1 µM) alone or together with FPS-ZM1 (3 µM) for 24 h, and analysed at 7 DIV when compact myelin is already observed. Slices were collected and stored in RIPA (radio-immunoprecipitation assay buffer) for protein extraction, TRIzol® reagent at 20 °C for RNA extraction, or fixed in 4% PFA for immunohistochemistry assays.

2.4. Immunochemistry and data analysis of primary oligodendrocyte cell culture

Fixed OPC were permeabilized with 1 nM HEPES, 2% heat-inactivated horse serum (Gibco), 10% FBS (Biochrom AG), 1% BSA (Sigma) and 0.25% Triton x100 (Roche Diagnostics, Indianapolis, USA) in Hank's Balanced Salt Solution (HBSS, Gibco). The percentage of OL in each differentiation stage was assessed by double immunocytochemical staining using antibodies directed to NG2 (rabbit, 1:200, Merck Millipore, Billerica, MA, USA) to identify the OPC and MBP (rat, 1:200, Serotec, Raleigh, NC, USA) for mature OL, with secondary Alexa Fluor 594 anti-rat and Alexa 488 anti-rabbit antibodies (1:1000, Invitrogen). To identify the total number of cells, nuclei were stained with Hoechst 33258 dye (1:1000, Sigma). Fluorescence was visualized using a Zeiss camera adapted to an AxioSkope® microscope (Zeiss, Germany). Pairs of U.V., red and green-fluorescence images of ten random microscopic fields (original magnification: 400x) were

acquired per sample. The number of $NG2^+$ and MBP^+ cells were counted in a minimum of 200 $Hoescht^+$ nuclei from at least four independent experiments and expressed as percentage *vs.* total cell number. To categorize the morphology of MBP^+ cells, three different stages were defined: cells with poorly branched processes (stage 1), cells with complex branched processes (stage 2) and cells with complex branched processes that partially form membranes (stage 3). To quantify the morphological complexity of the processes, first principal processes of interest were outlined using NeuronJ for ImageJ software to exclude adjacent cells. Subsequently, a minimum of 50 MBP^+ cells from each condition were analysed for the number of intersections made by branching processes with each successive circle using Sholl analysis for ImageJ. The program superimposes a grid of 20 concentric circles with increasing radius on an OL cell body (starting radius, 2 µm; step size, 2 µm; end, 80 µm).

2.5. Immunochemistry and data analysis of organotypic cerebellar slice cultures

After fixation, membranes containing cerebellar slices were incubated with blocking solution [1 nM HEPES, 2% heat-inactivated horse serum, 10% heat-inactivated FBS, 1% BSA (Sigma) and 0.25% Triton X-100 in HBSS] for 3 h at room temperature. Slices were then incubated with primary antibodies diluted in blocking solution for 24 h, at 4 °C. The following antibodies were used: neurofilament medium (NF-160, mouse, 1:200, Novocastra, Wetzlar, Germany) for neuronal axons, NG2 (rabbit, 1:200, Merck Millipore) for OPC, MBP (rat, 1:200, Serotec) for mature OL, GFAP (mouse, 1:200, Novocastra) for astrocytes, calcium-binding adapter molecule 1 (Iba1, rabbit, 1:250, WAKO) for microglia, S100B (rabbit, 1:200, Abcam, Cambridge, UK) and RAGE (rabbit, 1:100, Abcam). Then, slices were incubated with Alexa 594 anti-rat, Alexa 488 anti-mouse, Alexa 488 anti-rabbit (1:1000, Invitrogen) secondary antibodies in blocking solution for 24 h, at 4 °C. To identify the total number of cells, nuclei were stained with Hoechst 33258 dye (1:1000). Fluorescent images were acquired using a Confocal Point Scanning Microscope Zeiss LSM 710 META (Zeiss, Germany) using a 20/1.2 (zoom). Binary masks were defined using a cut-off intensity threshold value for each region of interest, which corresponds to a minimum intensity due to specific staining above background values. The number of $NG2^+$ and MBP^+ cells were counted in a minimum of 200 $Hoescht^+$ nuclei from at least four independent experiments and expressed as percentage *vs.* total cell number. The percentage of the area occupied by NF-160 and MBP was automatically calculated using ImageJ software. Regarding myelination, the percentage of myelinated fibers was obtained by the ratio between the area of co-localization of NF-160 and MBP and the total area occupied by NF-160. Results are given by averaging values determined in the separate microscopic fields from slices of different animals.

2.6. Gene expression

Total RNA was isolated from 7 DIV OL and treated slices using the TRIzol® reagent method according to the manufacturer's instructions (Invitrogen) and RNA concentration quantified using Nanodrop ND-100 Spectrophotometer (NanoDrop Technologies, Wilmington, DE, USA). Aliquots of 300 ng of total RNA were reversely transcribed using the SensiFASTTM cDNA Synthesis Kit

(Bioline, MA, USA), under recommended conditions. qRealTime- PCR was performed on a real-time PCR detection system (Applied Biosystems 7300 Fast Real-time PCR System, Applied Biosystem, Madrid, Spain) using a SensiFASTTM SYBR High-ROX Kit (Bioline). The qRealTime-PCR was performed in 96 well plates with each sample performed in duplicate and a non-template control was included for each gene. The sequences used as primers are listed in Supplementary Table II.1.

qRealTime-PCR was performed under optimized conditions: 50 °C for 2 min, 95 °C for 10 min followed by 40 cycles at 95 °C for 15 s and 62 °C for 1 min. In order to verify the specificity of the amplification, a melt-curve analysis was performed, immediately after the amplification protocol. Relative mRNA concentrations were calculated using the Pfaffl modification of the ΔΔCT equation, where CT is the cycle number at which fluorescence passes the threshold level of detection, taking into account the efficiencies of individual genes. The results were normalized to the housekeeping gene b-actin in the same sample and the initial amount of the template of each sample was determined as relative expression by the formula $2^{-\Delta\Delta CT}$. ΔCT is the value obtained for each sample by performing the difference between the mean CT value of each gene of interest and the mean CT value of b-actin. ΔΔCT of one sample is the difference between its ΔCT value and the ΔCT of the sample chosen as reference.

2.7. Western blot

Total protein extracts from slices were obtained by lysing cells in RIPA buffer, followed by sonication and centrifugation at 12,000 g for 10 min. Total protein concentrations were measured using the Bradford method with Bio-Rad's Protein Assay Reagent (Bio-Rad Laboratories, Hercules, CA, USA) and western blot was carried out as usual in our lab (Fernandes et al., 2007). Briefly, 50 µg of protein samples were subjected to SDS-PAGE and transferred to a nitrocellulose membrane. Membranes were blocked with 5% (w/v) non-fat dried milk, incubated with primary antibodies overnight at 4 °C [rabbit anti-NG2 (1:250), rat anti-MBP (1:250), rabbit anti-S100B (1:500), rabbit anti-RAGE (1:800), rabbit anti-pNF-κB (1:500, Abcam), rabbit anti-NF-κB (1:500, Santa Cruz Biotechnology), or mouse β-actin (1:10,000; Sigma)], and finally incubated with horseadish peroxidase-labelled secondary antibodies (1:5000) in non-fat dried milk, at room temperature during 1 h. Protein bands were detected using WesternBright Sirius reagent (Advansta, Menlo Park, CA, USA) and visualized using ChemiDocTM XRS System (Bio-Rad). Results were normalized to β-actin expression.

2.8. Statistical Analysis

All results are presented as mean ± SEM. Differences between two groups were determined by the two-tailed t-test performed on the basis of equal and unequal variance or by one-way ANOVA for multiple comparisons assuming a Gaussian distribution, using GraphPad PRISM 6.0 (GraphPad Software, San Diego, CA, USA), as appropriate. Statistical significance was defined as a p value <0.05.

3. Results

3.1. High S100B levels impair oligodendrocyte precursor differentiation and maturation

High levels of S100B have been identified in the serum/plasma of patients suffering from inflammatory conditions during the perinatal period that were associated with brain damage and myelin deficits (Huang et al., 2015; Zhou et al., 2015). Importantly, S100B was indicated as a predictor of poor prognosis associated with brain sequelae (Huang et al., 2015; Peng et al., 2017). So, we decided to explore the underlying mechanisms of S100B on oligodendrogenesis. Since S100B may have a dual role depending on the concentration (Sorci et al., 2010), we tested 10 nM and 1 μM to mimic either low or pathology-related high levels, respectively. So, pure rat OPC cultures were exposed to S100B during proliferation, e.g. first 24 h after seeding, 1st DIV, or upon the first 24 h of differentiation, 2nd DIV. After 7 DIV in differentiation medium, cells were immunostained for NG2 and MBP to calculate the number of OPC and mature OL, respectively. As demonstrated in Table II.1, 10 nM S100B had no effect on OL differentiation both during OPC proliferation or initial differentiation. Conversely, the highest concentration of S100B impaired oligodendrogenesis when incubated during the proliferation period, resulting in a significant reduction of mature MBP$^+$ cells (0.8-fold, $p<0.05$) with consequent increase of immature NG2$^+$ cells (1.6-fold, $p<0.05$). When OPC treatment occurred during initial differentiation, the effect had the same trend (0.8-fold MBP$^+$ and 1.7-fold NG2$^+$, $p<0.05$).

Table II.1. High levels of S100B reduce oligodendrocyte differentiation.

	Proliferation			Differentiation		
Cells	**Control**	**S100B 10 nM**	**S100B 1 μM**	**Control**	**S100B 10 nM**	**S100B 1 μM**
NG2$^+$ (%)	28.46±4.62	27.2±4.33	45.87±5.99*	21.25±4.48	22.22±4.29	34.84±4.63*
MBP$^+$ (%)	71.54±4.62	72.79±4.33	54.13±5.99*	78.75±4.48	77.79±4.29	65.16±4.63*

Primary oligodendrocyte precursor cells (OPC) were exposed to S100B (10 nM or 1 μM) during the initial 24 h of proliferation, or in the first 24 h of differentiation, and kept in differentiation medium to promote oligodendrocyte (OL) maturation until 7 days *in vitro.* Then OL were immunostained to identify OPC that express neural-glial antigen 2 (NG2) marker and mature OL that express myelin basic protein (MBP). Results are expressed as percentage of total number of cells. Values are mean ± SEM from at least four independent experiments. One-way ANOVA with Bonferroni multiple comparison test *$p<0.05$ *vs.* control.

Next to corroborate these results we decided to evaluate the gene expression of specific markers associated with OPC differentiation (Table 2). In accordance, we observed that physiological S100B had no significant effect on the expression of these markers, while high S100B levels markedly modulate them, namely when OPC were incubated during initial differentiation period. At this incubation time 1 μM S100B increased the gene expression of immature OPC markers such as PDGFRα and NG2 (1.8- and 1.7-fold, respectively, $p<0.05$), while decreased that of mature OL including MBP and PLP (0.7- and 0.6-fold, $p<0.05$ and $p<0.01$, respectively).

Since Olig1 and Olig2 transcription factors modulate oligodendrogenesis under physiological and pathological conditions (Ligon et al., 2006; Lin et al., 2006), their measurement was used to clarify the role of S100B at early and late steps of maturation. As indicated in Table II.2, exposure

to 1 μM S100B during differentiation decreased Olig1 (0.7-fold, $p<0.05$) and augmented Olig2 (2.0-fold, $p<0.01$) genes, suggesting once again an impaired oligodendrogenesis. Taken together, these results clearly show that exposure of OPC to high levels of S100B during proliferation or initial differentiation, processes that occurs during the perinatal period (Barateiro and Fernandes, 2014), impairs oligodendrogenesis.

Table II.2. High levels of S100B reduce gene expression of oligodendrocyte differentiation-related markers and increase that of immature ones namely during differentiation.

	Proliferation			Differentiation		
	Control	**S100B 10 nM**	**S100B 1 μM**	**Control**	**S100B 10 nM**	**S100B 1 μM**
PDGFR	1.00	1.00±0.10	1.10±0.11	1.00	0.85±0.10	1.77±0.27*
NG2	1.00	0.83±0.14	1.13±0.20	1.00	1.13±0.18	1.68±0.25*
MBP	1.00	0.98±0.21	0.85±0.19	1.00	0.99±0.12	0.72±0.06*
PLP	1.00	0.92±0.25	0.53±0.13*	1.00	0.92±0.09	0.63±0.09**
Olig1	1.00	1.36±0.14	0.81±0.19	1.00	1.06±0.12	0.68±0.06*
Olig2	1.00	0.98±0.21	1.30±0.05*	1.00	1.24±0.16	1.97±1.12**

Primary oligodendrocyte precursor cells (OPC) were exposed to S100B (10 nM or 1 μM) during the initial 24 h of proliferation, or in the first 24 h of differentiation, and kept in differentiation medium to promote oligodendrocyte (OL) maturation until 7 days *in vitro.* Gene expression of immature (PDGFRα/NG2) and mature (MBP/PLP) OL markers, and of transcription factors (Olig1/Olig2) were determined by qRealTime-PCR. Values are mean ± SEM from at least four independent experiments. One-way ANOVA with Bonferroni multiple comparison test ** $p<0.01$ and * $p<0.05$ *vs.* control.

3.2. RAGE antagonist prevents S100B-induced impairment of oligodendrocyte differentiation and morphological maturation

As our previous results point to a toxic effect of high levels of S100B on oligodendrogenesis, in particular at initial differentiation, we next sought to determine if these effects were mediated through RAGE engagement. For this purpose we used a specific RAGE antagonist, the FPS-ZM1, previously shown to prevent S100B binding to RAGE (Deane et al., 2012). Only the initial differentiation time point and the 1 μM concentration of S100B were considered. OL exposure to elevated concentrations of S100B upregulated mRNA expression of both S100B and RAGE (1.4-fold, $p<0.05$), as observed in Supplementary Figure II.1. In contrast, co-treatment of OL with S100B plus a FPS-ZM1 abrogated S100B-induced gene expression of both S100B and RAGE (0.6- and 1.0-fold, $p<0.05$, respectively) confirming that RAGE engagement is involved in S100B-RAGE axis expression promoting a further exacerbation of OL response to S100B.

As depicted in Figure II.1A and B, OPC co-incubation with S100B and FPS-ZM1 abrogated the S100B-induced impairment of the differentiation process by restoring the number of mature MBP^+ cells and immature $NG2^+$ cells to control values ($p<0.05$).

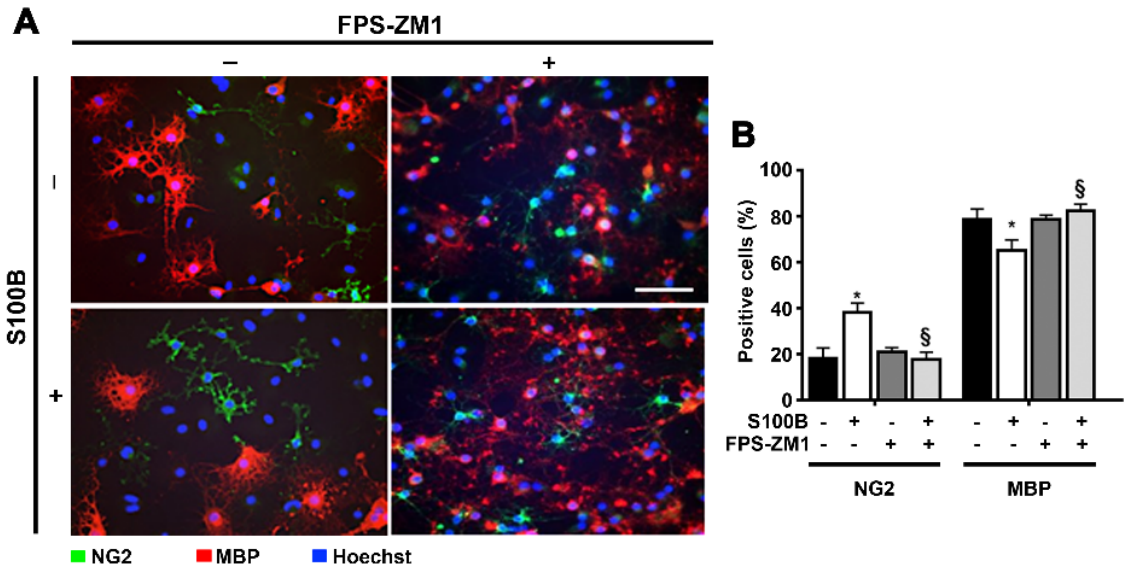


Figure II.1. Elevated S100B levels impair oligodendrocyte differentiation that is rescued by RAGE antagonist. Primary oligodendrocyte precursor cells (OPC) were exposed to S100B (1 µM) or S100B plus RAGE antagonist FPS-ZM1 in the first 24 h of differentiation, and kept in differentiation medium to promote oligodendrocyte (OL) maturation. After 7 days *in vitro* OL were immunostained to identify OPC that express NG2 (green), mature OL that express myelin basic protein (MBP) (red) and stained with Hoechst to detect nuclei (blue). Scale bar represents 50 µM. (**A**) Representative images of OL following treatment with S100B or S100B plus FPS-ZM1. (**B**) Quantification of the relative numbers of $NG2^+$ and MBP^+ cells. Values are mean ± SEM from at least four independent experiments. One-way ANOVA with Bonferroni multiple comparison test (*$p<0.05$ *vs.* control, and $^{§}p<0.05$ *vs.* S100B alone).

These results were supported by gene expression analysis of specific OL markers (Figure II.2). Indeed, co-incubation with S100B and FPS-ZM1 prevented S100B-induced increase of immature OPC markers (~65% for PDGFRα and >100% for NG2, $p<0.01$), as well as S100B-induced decrease of mature OL markers (>100% for MBP and ~50% for PLP, $p<0.01$ and $p<0.05$, respectively). FPS-ZM1 also prevented the S100B-induced gene expression of the oligodendogenesis-associated transcription factors (Figure II.2C), doubling Olig1 expression ($p<0.01$) and inhibiting Olig2 upregulation by ~40% ($p<0.01$).

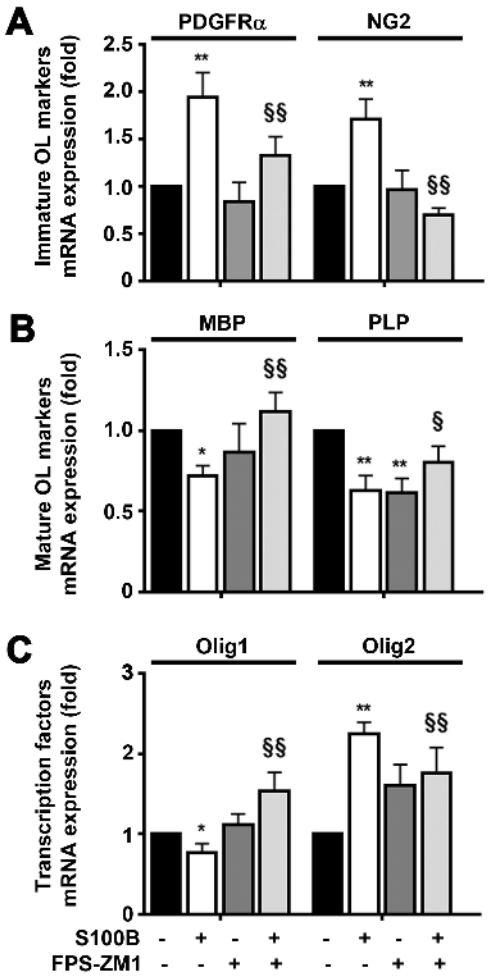


Figure II.2. Elevated S100B levels change the expression of oligodendrogenesis-associated genes in oligodendrocyte cultures, which is prevented by RAGE antagonist. Primary oligodendrocyte precursor cells (OPC) were exposed to S100B (1 µM) or S100B plus RAGE antagonist FPS-ZM1, in the first 24 h of differentiation, and kept in differentiation medium to promote oligodendrocyte (OL) maturation. Relative levels of the gene expression of the (**A**) immature (PDFGRα/NG2) and (**B**) mature (MBP/PLP) OL markers together with (**C**) transcription factors (Olig1/Olig2) were determined by qRealTime-PCR. Values are mean ± SEM from at least four independent experiments performed. One-way ANOVA with Bonferroni multiple comparison test (**p<0.01 and *p<0.05 *vs.* control, and §§p<0.01 and §p<0.05 *vs.* S100B alone).

Besides the expression of mature specific myelin proteins, the morphologic complexity and the formation of myelin membranes are important features of a proper OL differentiation. In this context, OPC incubation with non-physiological levels of S100B impaired the arborisation observed in mature OL at 7 DIV resulting in an increased number of MBP^+ cells in more immature stage 1 (2.0-fold, p<0.01), decreasing them in stage 2 (0.7-fold, p<0.05) with no cells presenting the more mature stage 3 morphology (Figure II.3A).

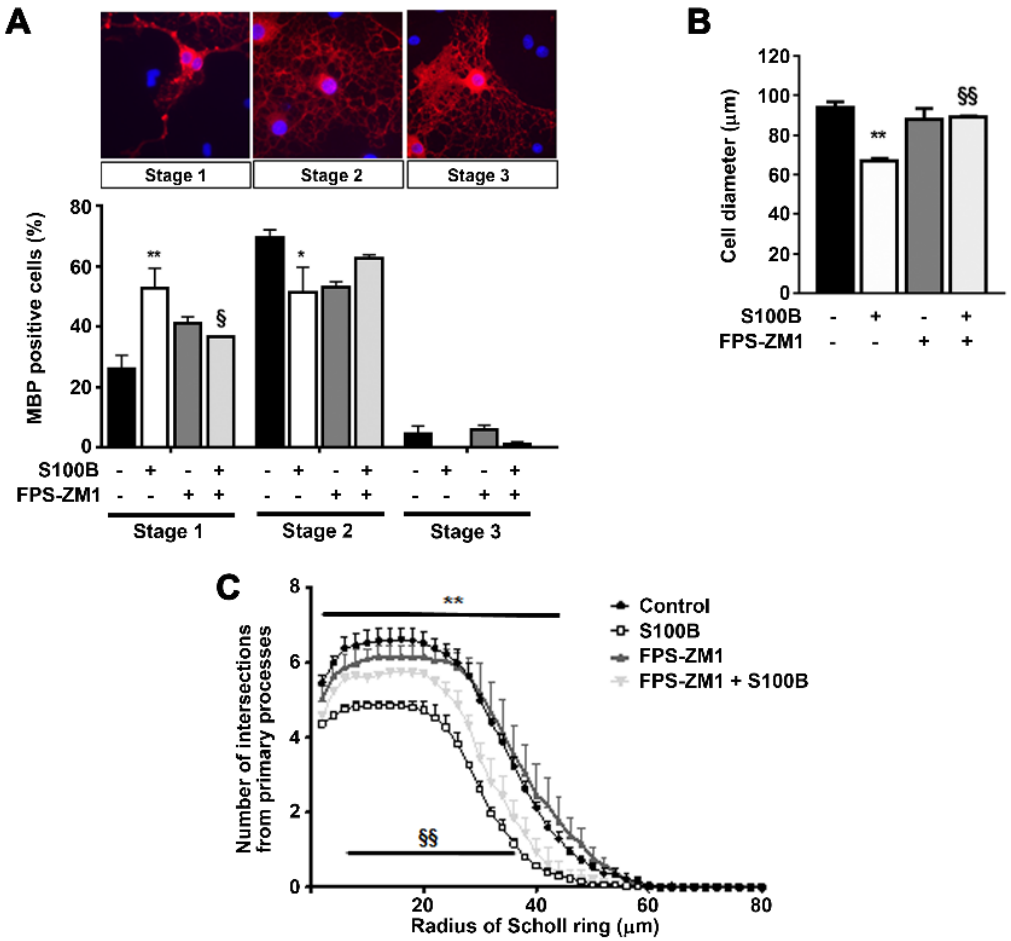


Figure II.3. Increased S100B levels impair oligodendrocyte morphological maturation, which is prevented by RAGE antagonist. Primary oligodendrocyte precursor cells (OPC) were exposed to S100B (1 µM) or S100B plus RAGE antagonist FPS-ZM1 in the first 24 h of differentiation, and kept in differentiation medium to promote oligodendrocyte (OL) maturation. After 7 days *in vitro* OL were immunostained for myelin basic protein (MBP) and (**A**) positive cells categorized according to their increasing morphologic maturity: cells with poorly branched processes (Stage 1), cells with complex branched processes (Stage 2) and cells with complex branched processes that partially form membranes (Stage 3). (**B**) MBP cells diameter was evaluated. (**C**) Sholl analysis of primary processes from at least 50 MBP^+ cells was assessed in each condition. Values are mean ± SEM from at least four independent experiments performed. One-way ANOVA with Bonferroni multiple comparison test (**p<0.01 and *p<0.05 *vs.* control, and §§p<0.01 and §p<0.05 vs S100B alone).

Interestingly, when OPC were treated with S100B in the presence of FPS-ZM1, these effects were prevented with a reduction of MBP^+ cells in stage 1 (~60%, p<0.01) and a tendency to increase MBP^+ cells in stage 2 (~60%). As observed in Figure II.3B, S100B treatment reduced cell diameter (0.7-fold, p<0.01), a feature that was abrogated by FPS-ZM1 co-incubation (p<0.01). Using Sholl analysis, we determined that S100B led to a decrease in mature OL ramifications, based on the reduction of the average number of intersections per Sholl ring for MBP^+ cells compared to control ones (from 2 to 44 µm radius, p<0.01) (Figure II.3C). The maximum length of mature OL processes was also decreased following exposure to S100B (from 64 to 58 mm). Interestingly, co-treatment with FPS-ZM1 prevented S100B-induced reduction of the average number of intersections per Sholl ring (from 14 to 34 µm radius, p<0.01), suggesting that RAGE

engagement have a key role in the deleterious effect of high S100B levels on the cytoskeleton reorganization that occurs during OL processes extension.

3.3. Organotypic cerebellar slice cultures exposed to high levels of S100B show marked expression of both S100B and RAGE, as well as of astrogliosis that are prevented by RAGE antagonist

Given our results on pure OL cultures and the role of S100B on neuronal and other glial cells (Sorci et al., 2010), namely concerning neurotoxicity and inflammatory response, we next decided to evaluate the role of S100B in OCSC. We started by immunostaining slices for S100B and RAGE to characterize the expression of these proteins under our experimental conditions. As observed in Figure II.4A, S100B expression is markedly induced upon S100B incubation with a cytoplasmatic staining mostly co-localizing with the astrocytic specific marker GFAP, suggesting its primary source. Interestingly, S100B also induced astrogliosis as observed by increased cell body of $GFAP^+$ astrocytes that co-localize with S100B expression. However, we may not exclude the expression of S100B by other cells, including OL since it was reported that the OL-93 cell line is able to release high levels of S100B under serum and glucose deprivation conditions (Steiner et al., 2008). In addition, RAGE expression was highly enhanced upon S100B treatment and curiously shifted its nuclear position in control slices to a cytoplasmic position upon S100B incubation (Figure II.4B). This RAGE expression could be appointed to several brain cell types including neurons, astrocytes or even microglia, as previously reported by us and others in human and animal models of neurodegenerative disorders (Barateiro et al., 2016a; Choi et al., 2014). Co-incubation with FPS-ZM1 not only reduced S100B and RAGE expression but also maintained RAGE expression at a nuclear level (Figure II.4A and B). However, astrocytes did not return to their original morphology, maintaining a more reactive and less branched phenotype than non-treated cultures, possibly indicating a partial action of S100B. On the other hand, S100B co-localization with astrocytes was markedly reduced, suggesting an accumulation of this protein at the extracellular space, given the partial blockage of its interaction with RAGE by FPS-ZM1 co-incubation.

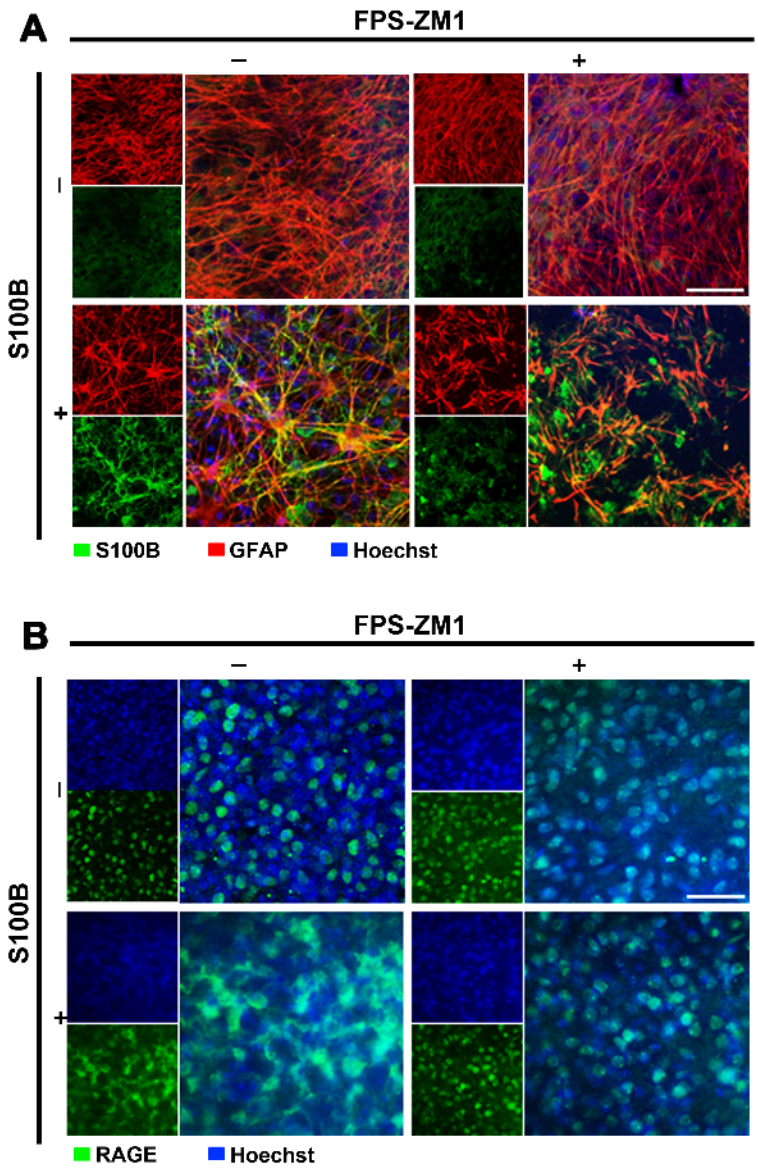


Figure II.4. Excessive extracellular S100B levels promote astrogliosis and increase S100B and RAGE immunoreactivity in organotypic cerebellar slice cultures, that is prevented by RAGE antagonist. Organotypic cerebellar slice cultures (OCSC) were exposed to S100B (1 μM) or S100B plus RAGE antagonist FPS-ZM1 at 3 days *in vitro* (DIV) for 24 h. At 7 DIV OCSC were immunostained for (**A**) S100B (green), astrocytes (GFAP) (red) or (**B**) immunostained for RAGE (green). Hoechst stain was used to detect nuclei (blue). Representative images are shown. Scale bar represents 50 μm.

Results were corroborated by protein and gene expression data (Figure II.5) of these markers. Exposure of OCSC to excessive S100B levels increased protein expression of both S100B and RAGE (1.5- and 1.8-fold, $p<0.05$, respectively) that was prevented by FPS-ZM1 co-incubation to levels comparable to control ones ($p<0.01$). Also gene expression was affected by S100B treatment upregulating S100B and RAGE (1.6-fold, $p<0.01$ and 1.7-fold, $p<0.05$, respectively), that once again was inhibited by FPS-ZM1 co-treatment (~60% for S100B and ~100% for RAGE, $p<0.01$). These results corroborate the previous OL data indicating that excessive S100B also enhances S100B-RAGE axis expression via RAGE engagement in these slice cultures.

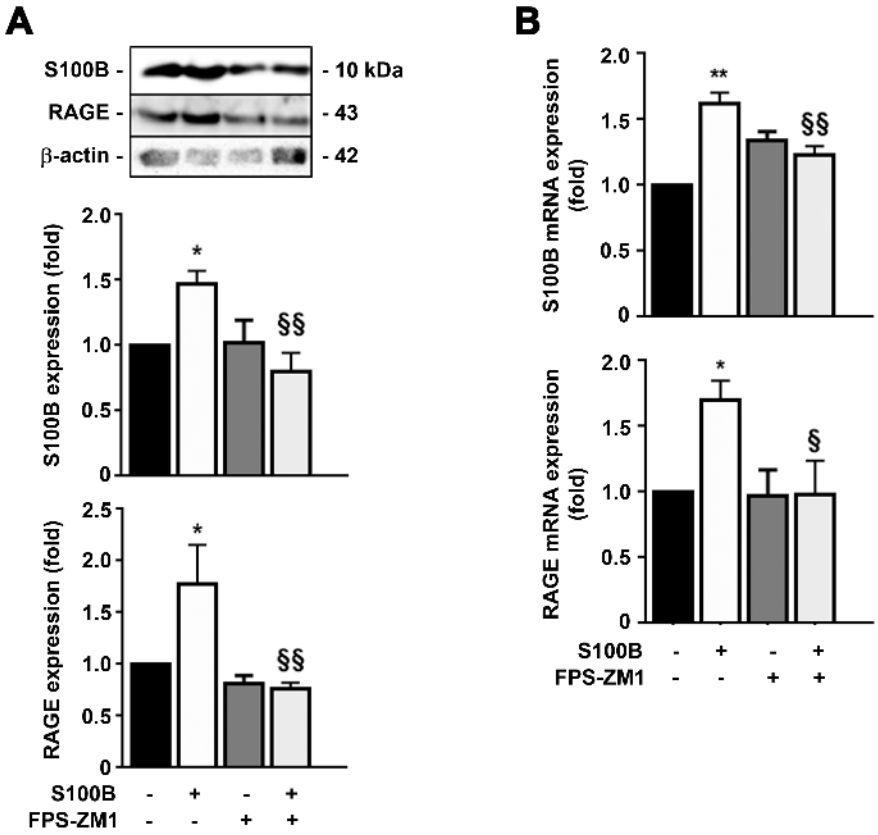


Figure II.5. Excessive extracellular S100B levels increase S100B and RAGE expression in organotypic cerebellar slice cultures, which are prevented by RAGE antagonist. Organotypic cerebellar slice cultures (OCSC) were exposed to S100B (1 μM) or S100B plus RAGE antagonist FPS-ZM1 at 3 days *in vitro* (DIV) for 24 h and allowed to recover until 7 DIV. (**A**) Protein expression of S100B and RAGE was evaluated by Western Blot. (**B**) Relative levels of S100B and RAGE gene expression were determined by qRealTime-pCR. Results are mean ± SEM from at least four independent experiments. One-way ANOVA with Bonferroni multiple comparison test (**p<0.01 and *p<0.05 vs. control, and §§p<0.01 and §p<0.05 vs S100B alone).

3.4. S100B-induced impairment of oligodendrocyte differentiation and myelination in organotypic cerebellar slice cultures is prevented by RAGE antagonist

Having observed the marked induction of S100B-RAGE axis we next evaluated the effect of high S100B levels on OL differentiation. When OCSC were treated with S100B we observed a significant reduction in the number of mature MBP^+ cells (0.8-fold, $p<0.05$) in parallel with an increase of immature $NG2^+$ cells (1.1-fold, $p<0.05$) (Figure II.6A and B). Co-incubation with RAGE antagonist FPS-ZM1 completely prevented this effect ($p<0.01$) and even favored a higher OL differentiation, increasing the number of MBP^+ cells (1.2- fold, $p<0.05$) and decreasing that of $NG2^+$ cells (0.8-fold, $p<0.05$), when compared to control values. The same pattern was observed when protein expression of MBP and NG2 was evaluated by Western blot (Figure II.6C). Indeed, S100B incubation decreased MBP expression (0.3-fold, $p<0.05$) and increased that of NG2 (1.6-fold, $p<0.05$). Co-treatment with FPS-ZM1 abrogated S100B-induced protein expression changes to levels comparable to control ones ($p<0.05$).

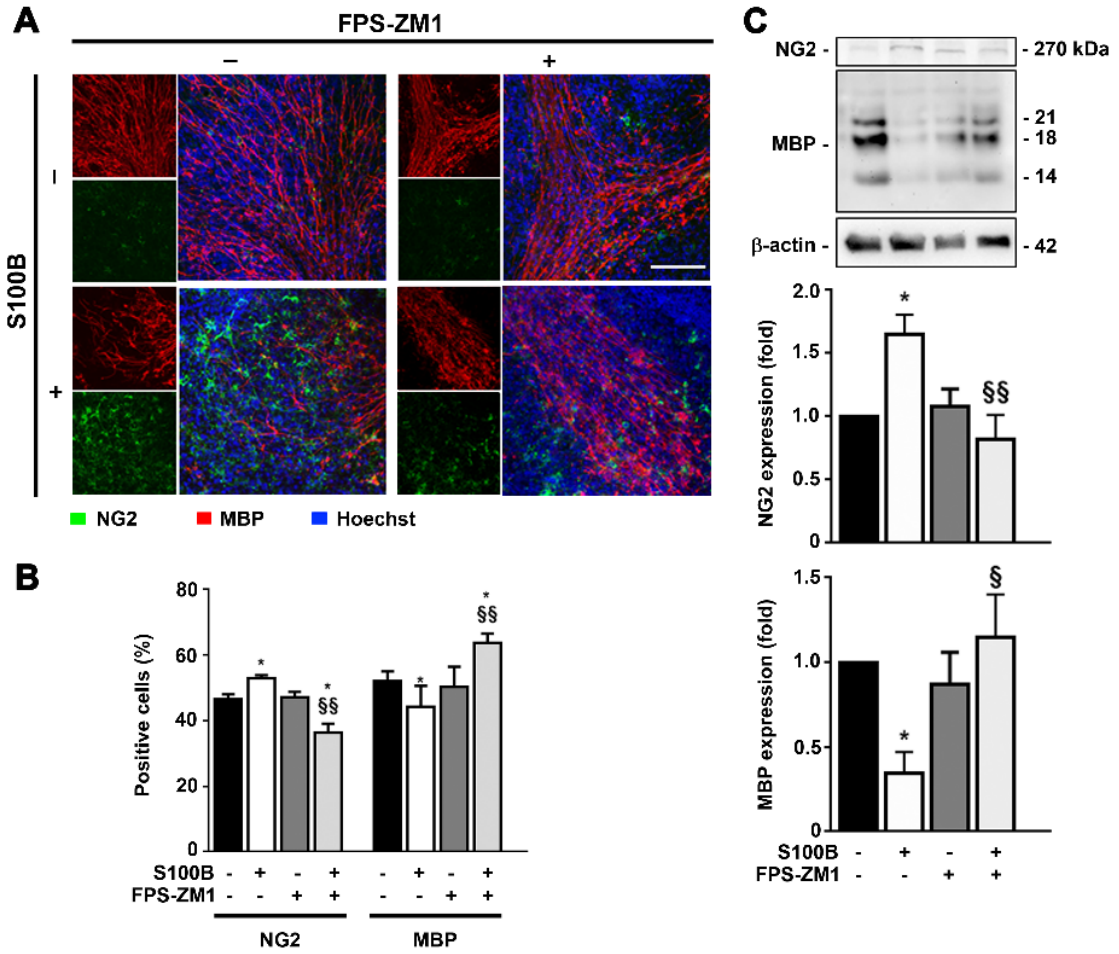


Figure II.6. High S100B levels cause oligodendrocyte maturation failure in organotypic cerebellar slice cultures that is prevented by RAGE antagonist. Organotypic cerebellar slice cultures (OCSC) were exposed to S100B (1 µM) or S100B plus RAGE antagonist FPS-ZM1 at 3 days *in vitro* (DIV) for 24 h. At 7 DIV OCSC were immunostained to identify immature oligodendrocytes (OL) that express NG2 marker (green) and mature OL that express myelin basic protein (MBP) (red) and stained with Hoechst to detect nuclei (blue). (**A**) Representative images are shown. Scale bar represents 100 µm. (**B**) Quantification of the relative numbers of $NG2^+$ and MBP^+ cells following S100B and/or RAGE antagonist FPS-ZM1 treatments was performed. (**C**) Protein expression of NG2 and MBP was evaluated by Western Blot. Results are mean ± SEM from at least four independent experiments. One-way ANOVA with Bonferroni multiple comparison test (*$p<0.05$ *vs.* control, and $^{§§}p<0.01$ and $^{§}p<0.05$ *vs.* S100B alone).

To further confirm these findings we evaluated gene expression of specific OL markers (Figure II.7) in our slice model. S100B treatment increased mRNA expression of PDGFRα (1.4-fold, $p<0.05$) and decreased that of PLP (0.7-fold, $p<0.05$), while no significant effects were observed for NG2 or MBP mRNA. Co-incubation of S100B with FPS-ZM1 almost completely prevented S100B effects ($p<0.05$, Figure II.7A and B). Interestingly, also the oligodendogenesis-associated transcription factors were affected by S100B exposure, based on the reduction of Olig1 (0.7-fold, $p<0.05$) and an increase of Olig2 mRNA expression (1.2-fold, $p<0.05$, Figure II.7C). Once again, co-treatment with FPS-ZM1 prevented S100B action and kept Olig1/Olig2 mRNA expression near control levels ($p<0.05$).

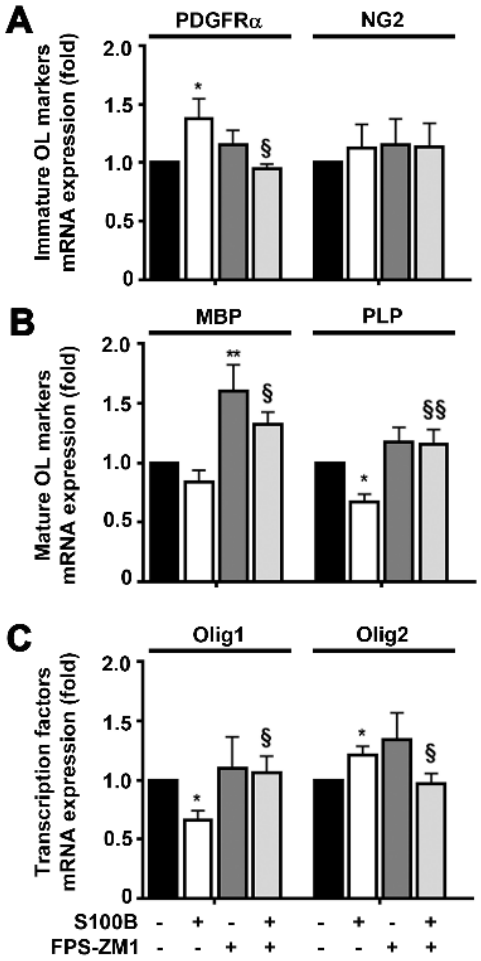


Figure II.7. Elevated S100B levels change the expression of oligodendrogenesis-associated genes in organotypic cerebellar cultures, which is prevented by RAGE antagonist. Organotypic cerebellar slice cultures (OCSC) were exposed to S100B (1 μM) or S100B plus FPS-ZM1 at 3 days *in vitro* (DIV) and allowed to recover until 7 DIV. Relative gene expression levels of (**A**) immature (PDFGRα/NG2) and (**B**) mature (MBP/PLP) OL markers, together with (**C**) transcription factors (Olig1/Olig2) were determined by qRealTime-PCR. Results are mean ± SEM from at least four independent experiments. One-way ANOVA with Bonferroni multiple comparison test (**p<0.01 and *p<0.05 *vs.* control, and §§p<0.01 and §p<0.05 *vs.* S100B alone).

Based on our oligodendrogenesis results we next assessed S100B effects on myelination. As shown in Figure II.8, our OCSC show ~90% of myelinated axons at 7 DIV, corroborating the value of this model to evaluate defects in neurodevelopmental myelination. As expected, high S100B levels exposure reduced the formation of myelinated tracts (0.8-fold, p<0.05) that was prevented by co-treatment with the RAGE antagonist FPS-ZM1 (p<0.05).

These results show that elevated levels of S100B during the neurodevelopmental myelination impair oligodendrogenesis, also delaying the differentiation into mature OL and the neurodevelopmental myelination through RAGE engagement.

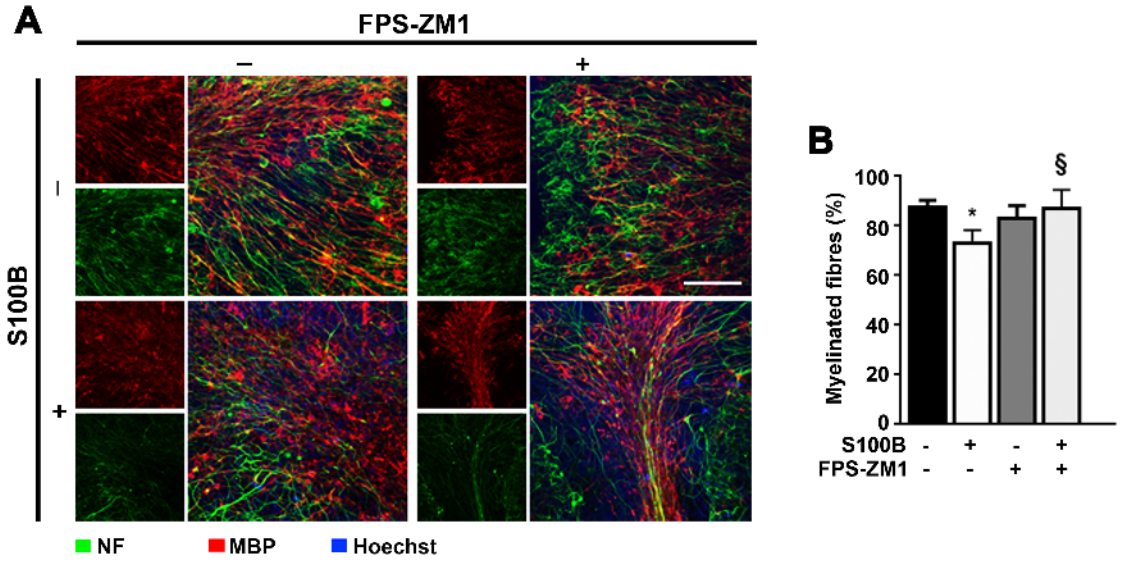


Figure II.8. Elevated S100B levels impair myelination in organotypic cerebellar slice cultures that is prevented by RAGE antagonist. Organotypic cerebellar slice cultures (OCSC) were exposed to S100B (1 μM) or S100B plus FPS-ZM1 at 3 days *in vitro* (DIV) for 24 h. At 7 DIV OCSC were immunostained for neuronal axons [neurofilament-160 (NF-160)] (green) and mature oligodendrocytes [myelin basic protein (MBP)] (red), and stained with Hoechst to detect nuclei (blue). (**A**) Representative images are shown. Scale bar represents 100 μm. (**B**) The percentage of myelinated fibres was calculated by the ratio between the area of co-localization of NF-160 and MBP and the total area occupied by NF-160. One-way ANOVA with Bonferroni multiple comparison test (*$p<0.05$ *vs.* control, and $^{§}p<0.05$ *vs.* S100B alone).

3.5. S100B-induced neuronal impairment in organotypic cerebellar slice cultures is prevented by RAGE antagonist

Micromolar S100B levels were shown to induce apoptosis in neuronal cells (Mariggio et al., 1994), while in N18 neuroblastoma cells this detrimental effect occurred via RAGE signalling in an oxidant dependent manner (Huttunen et al., 2000). So, S100B either through a direct effect or via myelination impairment may also affect axonal maintenance. Thus, we next evaluated the role of excessive S100B in axonal integrity and synaptic markers in our slice model. We observed that S100B decreased the total area of NF- 160 staining (0.7-fold, $p<0.01$, Figure II.9A), as well as the mRNA expression of the synaptic genes PSD-95 and synapthophysin (0.6-fold, $p<0.01$) (Figure II.9B and C). However, when slices were treated with S100B in the presence of FPS-ZM1 neuronal integrity was preserved (~95%, $p<0.01$) and the loss of synaptic markers prevented (~70% for PSD-95, $p<0.05$ and ~95% for synapthophysin, $p<0.01$).

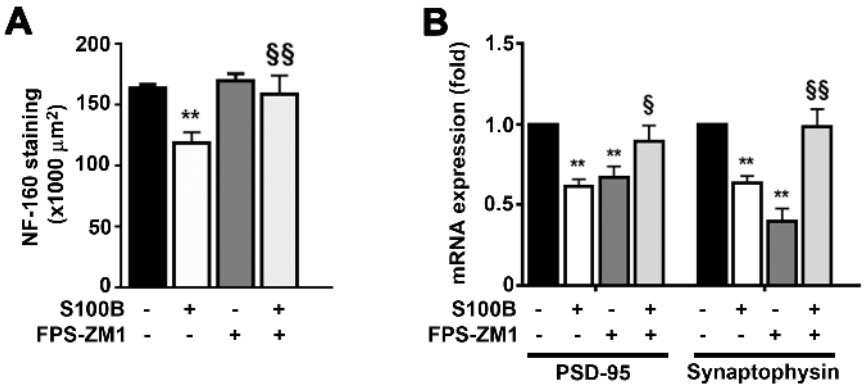


Figure II.9. Elevated S100B levels impair neuronal integrity in organotypic cerebellar slice cultures that is prevented by RAGE antagonist. Organotypic cerebellar slice cultures (OCSC) were exposed to S100B (1 µM) or S100B plus FPS-ZM1 at 3 days *in vitro* (DIV) for 24 h. (**A**) At 7 DIV OCSC were immunostained for neuronal axons [neurofilament-160 (NF-160)] and axon integrity was obtained by averaging the area occupied by NF-160 staining. (**B**) Relative levels of synaptic markers (PSD-95 and synaptophysin) were determined by qRealTime-PCR. Results are mean ± SEM from at least four independent experiments. One-way ANOVA with Bonferroni multiple comparison test (**p<0.01 *vs.* control, and §§p<0.01 and §p<0.05 *vs.* S100B alone).

Overall, these results indicate that S100B-RAGE axis may be promoting both OL and neuronal impairment, with consequent myelination and synaptogenesis failure.

3.6. S100B-induced inflammation in organotypic cerebellar slice cultures is prevented by RAGE antagonist

As mentioned above, S100B may activate glial cells leading to an inflammatory response (Bianchi et al., 2010; Villarreal et al., 2014). Indeed, we observed a marked astrogliosis in our culture model (Figure II.4). Thus, we decided to evaluate how S100B-RAGE axis could be modulating inflammation in OCSC. First, we assessed NF-κB activation by determining the ratio between pNF-κB and total NF-κB by Western Blot. Although no differences were found for total NF-κB (Figure II.10A), pNF-κB increased following S100B treatment leading to a high pNF-κB/NF-κB ratio (3.0-fold, p<0.01). Co-exposure to RAGE antagonist FPS-ZM1 abrogated the S100B-induced NF-κB activation (p<0.01), as observed by a reduction in pNF-κB/NF-κB ratio. The alarmin high mobility group box 1 (HMGB1) is usually involved in an inflammatory response having a proinflammatory role also through RAGE binding and NF-κB signalling (Luan et al., 2010). Our results showed that elevated S100B increases HMGB1 mRNA expression (1.6-fold, p<0.01), which was abrogated upon FPS-ZM1 co-treatment (p< 0.01, Figure II.10B).

Release of first line proinflammatory cytokines, including tumor necrosis factor (TNF)-α and interleukin (IL)-1β, occurs downstream NF-κB activation, so we next assessed gene expression of such cytokines. As indicated in Figure II.10C, treatment of organotypic cerebellar slice cultures with S100B clearly induced both TNF-α and IL-1β mRNA expression (1.5-fold, p<0.05 and 1.7-fold, p<0.01, respectively). Again, co-exposure with FPS-ZM1 completely prevented this inflammatory response (p<0.01).

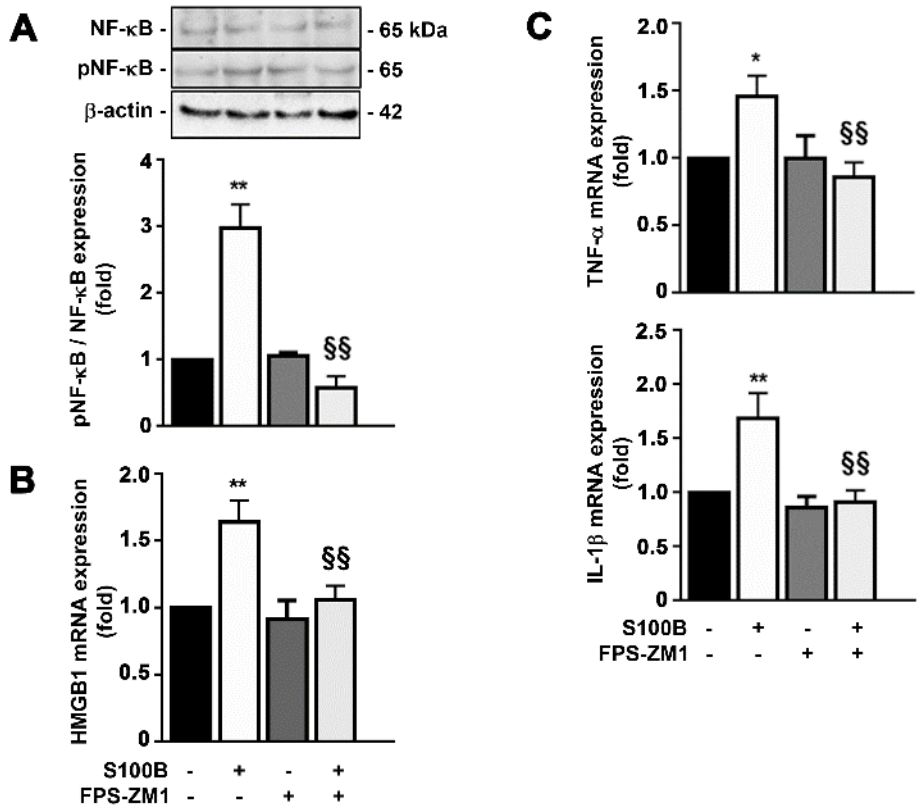


Figure II.10. Elevated S100B levels activate NF-κB and elicit TNF-α and IL-1β gene expression in organotypic cerebellar slice cultures, which are prevented by RAGE antagonist. Organotypic cerebellar slice cultures (OCSC) were exposed to S100B (1 μM) or S100B plus FPS-ZM1 at 3 days *in vitro* (DIV) for 24 h and allowed to recover until 7 DIV. (**A**) Protein expression of NF-κB and pNF-κB was evaluated by Western Blot. Gene expression of HMGB1 (**B**), TNF-α and IL-1β (**C**) was evaluated by qRealTime-PCR. Results are mean ± SEM from at least four independent experiments. One-way ANOVA with Bonferroni multiple comparison test (**p<0.01 and *p<0.05 *vs.* control, and §§p<0.01 *vs.* S100B alone).

Overall, our studies demonstrate that excessive S100B promotes a neuroinflammatory milieu, via RAGE engagement, in parallel with impaired neuronal connectivity and myelination, which may justify the observed neurotoxicity and myelination deficits observed in CNS inflammatory disorders during the perinatal period.

4. Discussion

Brain injury in the premature infant is associated with a high risk of neurodevelopmental disabilities and it primarily involves cerebral white matter damage since the developing OL are one of the main targets. In fact, recent evidences have been linking myelin abnormalities and white matter damage, described during the peri- and neonatal periods, to impaired OL maturation resulting thereby in their reduced capacity to synthesize proper myelin (Tolcos et al., 2017). During these stages of brain development, the impairment of white matter formation is potentiated, since vulnerable OPC and pre-myelinating OL are the predominating cells of the OL lineage (Back et al., 2001). Interestingly, it was demonstrated the presence of μM concentrations of S100B in the blood, urine and serum samples of patients with white matter damage in

conditions such as hypoxic-ischemic encephalopathy, intraventricular haemorrhage and PVL when comparing to those with no white matter injury (Huang et al., 2015; Zhou et al., 2015). Further studies even associated these high S100B levels to a poor prognosis of premature infants with PVL (Huang et al., 2015) and to a sustained brain injury of preterm infants with ischemic brain damage (Chiang et al., 2015), being a sign of adverse outcomes in children with CNS infections (Peng et al., 2017). So, it becomes crucial to understand the role of excessive S100B during neurodevelopment. Here, we used *in vitro* and *ex vivo* models to assess if: (1) high levels of S100B directly affect oligodendrogenesis; (2) whether in a more complex multicellular environment excessive S100B impairs oligodendrogenesis, *de novo* myelination, neuronal integrity and inflammation; and if (3) S100B-RAGE axis plays a role in S100B effects.

It has been described that S100B is expressed by OPC when they are committed to differentiate into mature OL, both in developing and adult mice brain (Deloulme et al., 2004), as well as in mature myelinating OL in brain and spinal cord (Hachem et al., 2005). Moreover, in an *in vivo* model lacking S100B, the OPC maturation was shown to be delayed following a demyelinating challenge (Deloulme et al., 2004). Later studies showed that stressed oligodendrocytic OLN-93 cell line not only express high intracellular S100B, but also its receptor RAGE (Steiner et al., 2008). More recently, we and others related S100B expression with white matter injury in multiple sclerosis (Barateiro et al., 2016a) and schizophrenia (Milleit et al., 2016). Indeed, we have observed a basal gene expression of both S100B and its receptor RAGE in our primary cultures of OL that was increased following treatment with μM concentrations of S100B. Further, our results clearly show that pathological elevated levels of S100B (1 μM), but not physiological ones (10 nM), increase the number of immature $NG2^+$ cells following the differentiation process indicating a delayed or impaired OPC differentiation into mature OL. These results were corroborated in our OCSC model where high S100B levels also impaired oligodendrogenesis. Data are in line with previous studies that observed OL maturation arrest and differentiation failure in chronic perinatal white matter injury (Segovia et al., 2008) and in PVL (Billiards et al., 2008), conditions where S100B is highly elevated (Chiang et al., 2015; Huang et al., 2015). Further, in parallel studies we have observed that following OCSC demyelination, where we showed that S100B was markedly expressed and released (Barateiro et al., 2016a), there is an increase of $NG2^+$ cells that is intensified by blockade of S100B with a specific antibody (data not shown). These results suggest that although elevated S100B levels may be detrimental for oligodendrogenesis, physiological S100B values may favor OPC proliferation and further differentiation for remyelination. Curiously, a recent study showed that astrocytes improve oligodendrogenesis following white matter damage through the release of the brain-derived neurotrophic factor (Miyamoto et al., 2015). However, in our *in vitro* system we used pure OL cultures with no possible astroglial source for this factor, while in the OCSC model the S100B-induced astrogliosis may be preventing the more neurotrophic role of astrocytes.

During myelination, OPC differentiation into mature OL is orchestrated by a complex program that uses several factors to control the proper timing of OPC migration, proliferation and differentiation (Barateiro et al., 2016b). Among these factors the transcription factors from the Olig

family, named by their high expression in cells of the oligodendroglial lineage (Zhou et al., 2000), have a major role in the myelination process (Meijer et al., 2012). Indeed, Olig1 reaches maximal expression during myelin membrane formation and is essential in the final stages of myelin production (Copray et al., 2006; Lu et al., 2002). In contrast, Olig2 is located in the OPC nucleus and not present along process outgrowth and membrane compaction (Othman et al., 2011). We observed a downregulation of Olig1 and upregulation of Olig2 mRNA in both OL and OCSC cultures by high S100B concentrations, what supports the OL differentiation and maturation arrest observed in these experimental models. Interestingly, it has been described a reduction of Olig1 expression in an animal model of neonatal PVL following hypoxia-ischemia, and associated with the inhibition of OL maturation and myelin formation (Cheng et al., 2015). Others showed that Olig1 is required for OL maturation and myelination following neonatal stroke (Sabo et al., 2017). On the other hand, PVL induction by N-methyl-D-aspartate intracerebral injection leads to a reduction of Olig2 mRNA in OPC from the corpus callosum but a marked increase in the sub-ventricular zone, suggesting the activation of OPC migration to the lesion area (Espinosa-Jeffrey et al., 2013). Our results corroborate these findings adding S100B as a new potential player in PVL-associated white matter injury. Although no direct effect of S100B/RAGE axis on Olig1 or Olig2 expression has been reported, it has been described that Olig1 plays a crucial role in transforming growth factor (TGF)-β induced cell motility (Motizuki et al., 2013), while a recent study showed the existence of an inhibitory cross-talk mechanism between TGF-β and RAGE signalling (Serban et al., 2016). So, it is possible that in our model activation of S100B/RAGE axis may be inhibiting TGF-β-Olig1 action on cell dynamics and therefore compromise morphological changes associated with oligodendrogenesis.

Throughout development, OL undergo process extension and arborisation until complete myelination. In our study, S100B treatment resulted in decreased OL morphological maturation, namely by the retraction of ramifications extension and membrane formation resulting in a reduced diameter and branching. Besides, in our OCSC model high S100B levels also promoted a decrease of the percentage of myelinated fibres and led to a retention of MBP protein in the OL soma. During oligodendrogenesis, MBP is initially found within OL nucleus and lately incorporated in the myelin sheath once myelination begins (Pedraza et al., 1997). In accordance with our results, a subset of cases of PVL in premature infants showed myelin abnormalities despite the similar MBP^+ cell density between PVL and age-matched controL (Billiards et al., 2008). In these PVL cases, it was observed an increased number of OL with perikaryal MBP immunostaining in contrast with a more distal cytoplasmic process expression seen in control samples. These evidences suggest a deficient ability of PVL OL to extend their processes to contact the axon and form a proper myelin sheath wrapping. In addition, in the same PVL samples the authors identified $O4^+$ OPC lacking processes, while they were found intact in control cases (Billiards et al., 2008). Curiously, we also observed a reduction of OPC diameter in the presence of μM concentration of S100B (data not shown).

Changes in OL process extension and impairment of morphological differentiation of pre-myelinating OL have been reported following inflammatory cytokine exposure, and to be

preventable by the use of corticosteroids (Mann et al., 2008). Additionally, brains of preterm babies and infants with white matter injury present high levels of proinflammatory cytokines (Kadhim et al., 2001, 2002), as well as transient gliosis (Haynes et al., 2003; Verney et al., 2012). Further, *in vivo* exogenous administration of these cytokines in early postnatal life resulted in the reduction of both the number and the diameter of myelinated axons, together with an increase in the density of OPC, indicating that cytokines impair myelination (Favrais et al., 2011). In our organotypic model S100B elicited a marked astrogliosis with the induction of an inflammatory response, what may have contributed to the reduced morphological complexity of OL, as well as to the decreased myelinated fibres we observed in this condition.

It is well established that communication between axons and OL is crucial for myelin sheath formation (Simons and Trajkovic, 2006). Not only the active role of axonal signals are needed to promote myelination, but also the homeostasis of myelinated axons is sustained by trophic and supporting factors released by myelinating cells (Tomassy et al., 2016). Therefore, delayed or incorrect myelination at critical periods during development could negatively affect the formation of neuronal networks and their synaptic integrity (Hagmann et al., 2010). Indeed, our results show that neuronal loss and downregulation of the pre- and post-synaptic markers mRNA expression occurs following S100B treatment due, at least in part, to myelination failure. Diffuse axonal damage is frequently observed in PVL cases of premature infants (Haynes et al., 2008) and associated with ischemia and inflammation (Volpe, 2009). Accordingly, excessive extracellular levels of S100B have been shown to induce neuronal dysfunction or apoptosis in a direct action (Mariggio et al., 1994), or as a result of gliosis with the release of nitric oxide and proinflammatory cytokines (Hu et al., 1997; Koppal et al., 2001). So, it is possible that the neuronal impairment seen in our OCSC model following S100B treatment may be a sum of impaired myelination, direct neurotoxicity and glial mediated neuronal damage.

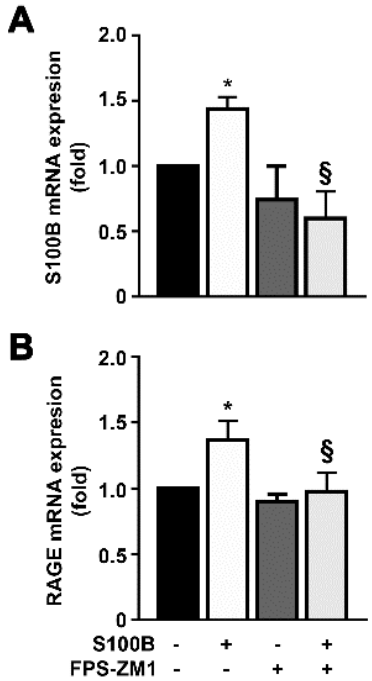
Upon secretion, S100B exerts autocrine and paracrine actions mainly via RAGE engagement (Donato, 2007), although binding of S100B to other receptors such as toll-like receptor (Tsoporis et al., 2012) and basic fibroblast growth factor receptor 1 (Riuzzi et al., 2011), have also been described. In human brain, S100B is mainly present as a dimer or tetramer and it is in a tetrameric form that S100B binds to RAGE with higher affinity (Ostendorp et al., 2007). Interestingly, it was shown that S100B promotes astrocyte migration by directly binding Src kinase and activating the PI3K/Akt and PI3K/RhoA pathways (Brozzi et al., 2009) or microglia migration via RAGE engagement and activation of Rac1-Cdc42 signalling (Bianchi et al., 2011). Pathways also involved in OL survival and myelination (Laursen et al., 2009). While S100B binding to RAGE was described as being crucial for nerve regeneration after sciatic nerve crush (Rong et al., 2004), the role of S100B-RAGE axis on CNS myelination is not clear. Although we have demonstrated that neutralization of S100B reduces myelin damage upon a demyelinating insult (Barateiro et al., 2016a), to our knowledge only two studies have addressed the role of RAGE in demyelination using *in vivo* models of experimental autoimmune encephalomyelitis (Yan et al., 2003) and intracerebral haemorrhage (Yang et al., 2015). In the first study the authors showed a marked reduction in the clinical score of the disease severity upon RAGE blockade, suggesting a reduced

demyelination, but no data concerning myelin are given (Yan et al., 2003). On the second study, the authors demonstrated that the same RAGE antagonist that we used in our study, the FPS-ZM1, reduced perihematomal nerve fibre injury (Yang et al., 2015). Here, we clearly show that RAGE engagement by excessive S100B impairs not only OPC differentiation and OL morphological maturation, but also axonal integrity and myelination since these effects were prevented by the use of the specific RAGE antagonist FPS-ZM1.

S100B acts as a proinflammatory protein, also through RAGE engagement, when present in elevated levels. Indeed, S100B not only regulates astrocyte shape (Brozzi et al., 2009), but also activates astrocyte and microglia in a RAGE-dependent manner leading to the release of inflammatory cytokines (Bianchi et al., 2007; Villarreal et al., 2014) via the activation of NF-κB (Bianchi et al., 2010; Villarreal et al., 2014). Our OCSC data clearly show astrogliosis and elevated S100B and RAGE expression upon treatment with high levels of S100B, suggesting a direct action on astrocyte activation. It has been shown that S100B is mainly constitutively released by astrocytes and its expression is regulated by several factors including TNF-α and IL-1β (Edwards and Robinson, 2006), while RAGE is upregulated by extracellular S100B and potentiates the induction of proinflammatory genes (Donato et al., 2013) creating an inflammatory loop. In accordance, we showed that S100B-induced NF-κB activation and gene expression of TNF-α and IL-1β are prevented upon co-incubation with the RAGE antagonist FPS-ZM1, corroborating the role of S100B-RAGE axis on the creation of an inflammatory milieu in our experimental model. On the other hand, we may not exclude that other inflammatory molecules that also bind RAGE, namely HMGB1, may also be indirectly involved in this process. Indeed, in a previous study we showed that upon demyelination, where S100B is released, there was an increase of HMGB1 mRNA expression that was prevented when S100B was neutralized with a specific antibody (Barateiro et al., 2016a). Similarly, in the present study, we observed that HMGB1 was highly expressed upon S100B incubation, and in this case may also bind RAGE to exacerbate initial S100B effect. Further, since the increase of HMGB1 mRNA levels was prevented by co-incubation with the FPS-ZM1, it seems that HMGB1 expression occurs downstream of RAGE engagement. This continuous amplification and perpetuation of inflammation may then contribute to the OL and neuronal damage observed in perinatal inflammatory conditions, and underlie long-term brain sequelae, or even the emergence of other CNS disorders along life. Accordingly, it was shown that even moderate inflammation, causes long-lasting myelination deficits, neuroarchitectural modifications and cognitive defects that extend into adult life (van Tilborg et al., 2016).

Altogether, these results indicate that excessive extracellular S100B levels impairs proper neurodevelopmental oligodendrogenesis and myelin formation, as well as neuronal integrity in an inflammatory environment, pointing to S100B as a surrogate biomarker of brain injury in perinatal conditions associated with white matter injury. We further demonstrated that these S100B effects are mediated in a great extend via RAGE engagement, suggesting that modulation of S100B-RAGE interaction may have a crucial beneficial role to prevent alterations of brain development by neonatal injuries.

5. Supplementary Material



Supplementary Figure II.1. Elevated S100B levels increase S100B and RAGE gene expression in primary oligodendrocyte cell cultures, which are prevented by RAGE antagonist. Primary oligodendrocyte precursor cells (OPC) were exposed to S100B (1 μM) or S100B plus RAGE antagonist FPS-ZM1, in the first 24 h of differentiation, and kept in differentiation medium to promote oligodendrocyte (OL) maturation. Gene expression of S100B (**A**) and RAGE (**B**) was evaluated by qRealTime-PCR. Results are mean ± SEM from at least four independent experiments. One-way ANOVA with Bonferroni multiple comparison test (*$p<0.05$ *vs.* control, and $^{§}p<0.05$ *vs.* S100B alone).

Supplemental Table II.1 - List of pairs of primers used for gene expression.

Gene	Forward	Reverse
HMGB1	ctcagagaggtggaagaccatgt	gggatgtaggttttcatttctctttc
IL-1β	cagagctgcgcttgcagag	gtcagcagccggttaccaag
MBP	tcgtcttcacgttcggtctg	ctcaaggccaggttcaggag
NG2	ctcacgaatcccagtggctt	tttctccttcgggtcagcac
Olig1	gcccaggccacgagtacaaa	tccactccgaaacccaacga
Olig2	gaaatggaataatcccgaactact	cccctcccaaataactcaaac
PDGFRα	acgttcaagaccagcgagtt	cagtttgatggacgggagtt
PLP	gccaggtcctggaacctttc	ggagcagggagatgcagatgag
PSD-95	cgaggatgccgtggcagcc	catggctgtggggtagtcagtgcc
RAGE	tgggcaccatcttcatcattc	ggtcacccagcacaccactt
S100B	acccacatctggcagaatgag	agccatgacctttcgcattag
Synaptophysin	tcaggactcaacacctcagtgg	aacacgaaccataagttgccaa
TNF-α	agcttacaacaggccaggtttc	cggctggcgacatacagtac
β-actin	Gctccggcatgtgcaa	aggatcttcatgaggtagt

IL-1β, interleukine-1β; MBP, myelin basic protein; NG2, neural-glial antigen 2; PDGFRα, platelet-derived growth factor receptor α; PLP, myelin proteolipid protein; RAGE, receptor for advanced glycation endproducts; TNF-α, tumor necrosis factor-α.

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Chapter III

Involvement of S100B-RAGE axis on organotypic cerebellar slice culture demyelination: relevance for multiple sclerosis pathogenesis

Gisela Santos[1], Andreia Barateiro[1], Dora Brites[1,2], Adelaide Fernandes[1,2*]

[1]Neuron Glia Biology in Health and Disease, Research Institute for Medicines (iMed.ULisboa), Faculty of Pharmacy, Universidade de Lisboa, Lisboa, Portugal;

[2]Department of Biochemistry and Human Biology, Faculty of Pharmacy, Universidade de Lisboa, Lisboa, Portugal.

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Abstract

Background

Increased expression of S100B and its specific receptor for advanced glycation end products (RAGE) has been described in multiple sclerosis (MS) patient samples, being associated with an active demyelinating process. We previously showed that a direct neutralization of S100B prevents demyelination and inflammatory response, improving oligodendrocyte maturation and remyelination. However, whether S100B actions occur through RAGE is not clarified.

Objective

To evaluate the role of S100B-RAGE axis in the course of a demyelinating insult.

Methods

Organotypic cerebellar slice cultures were demyelinated with lysophosphatidylcholine (LPC) with or without RAGE antagonist FPS-ZM1. The effect of S100B-RAGE axis on demyelination, neuronal function, oligodendrogenesis, glia reactivity and inflammation was determined by immunohistochemistry, western blot and qRealTime PCR.

Results

LPC-induced demyelination increased S100B/RAGE expression, in parallel with higher expression of markers of immature oligodendrocytes, compromised neuronal networks, and a marked gliosis with augmented inflammatory response. Interestingly, the use of RAGE antagonist FPS-ZM1 prevented LPC-induced demyelination, neuronal impairment and inflammation.

Conclusion

Our data implicate S100B-RAGE axis in the extent of myelin and neuronal damage, as well as of the inflammatory response that follows a demyelinating insult. These results demonstrate that blockade of S100B-RAGE axis may potentially prevent CNS damage and improve recovery in the course of MS.

Keywords: demyelination, inflammation, neurodegeneration, oligodendrogenesis, receptor for advanced glycation endproducts (RAGE), S100B

1. Introduction

Multiple sclerosis (MS) is a chronic inflammatory disease of the central nervous system (CNS) with a neurodegenerative component characterized by focal demyelinated areas, extensive axonal injury (Bjartmar et al. 2001), neuronal loss (Filippi and Rocca 2005), and synaptic alterations (Centonze et al. 2009, Rossi et al. 2010). In MS early inflammatory stages, activated immune cells highly produce proinflammatory factors including interleukin (IL)-1β and tumor necrosis factor (TNF)-α, which are not only cytotoxic but also induce glial reactivity leading to a further release of inflammatory factors, including the astroglial S100B protein.

S100B is a small Ca^{2+} binding protein highly expressed in the CNS, namely by astrocytes, that exerts both intra- and extracellular roles. Within cells, S100B is involved in the regulation of both proliferation and differentiation of neurons and glia (Donato et al. 2009, Sorci et al. 2010). Extracellularly S100B has a dual function in a concentration-dependent manner (Van Eldik and Wainwright 2003), with additional evidences suggesting also a multimeric-dependent S100B binding to the receptor for advanced glycation endproducts (RAGE) (Ostendorp et al. 2007). At physiological levels, in the nM range, S100B promotes cell proliferation and migration, while inhibits apoptosis and differentiation (Sorci et al. 2010). Indeed, S100B modulates neurite outgrowth, synaptogenesis and long-term plasticity (Arcuri et al. 2005), prevents astrocytic (Brozzi et al. 2009) and microglial activation and consequent inflammation (Zhang et al. 2011). Notwithstanding, elevated S100B concentrations, in the μM range, exacerbate the inflammatory response through glial activation and subsequent release of inflammatory cytokines and stress-related enzymes culminating in cell dysfunction and death (Bianchi et al. 2010, Sorci et al. 2010, Astrand et al. 2013).

Concerning oligodendrogenesis, S100B is expressed by oligodendrocyte precursor cells (OPC) and needed for their differentiation and maturation in myelinating oligodendrocytes (OL). In fact, S100B absence impairs the morphologic maturation of preOL cells *in vitro,* whereas $S100B^{-/-}$ mice showed a delayed OPC maturation following a demyelinating insult (Deloulme et al. 2004). Besides, S100B is associated with microtubular structures in cultured OL (LoPresti 2002), being implicated in their cytoskeleton dynamics (Richter-Landsberg and Heinrich 1995), which is critical for correct myelination. In fact, increased S100B levels in the brain have been associated with numerous disorders with altered myelination. Indeed, we reported the presence of excessive S100B in the cerebrospinal fluid (CSF) and serum of MS patients at diagnosis, as well as in active lesions of post-mortem brain samples (Barateiro et al. 2016). Additionally, S100B levels have been suggested to stratify the different stages of the disease, being the highest concentrations found in relapsing-remitting MS patients in opposite to lowest levels in progressive MS (Petzold et al. 2002, Bartosik-Psujek et al. 2011, Barateiro et al. 2016). S100B has also been indicated to monitor treatment efficacy, since S100B concentrations significantly decrease after therapy (Petzold et al. 2002, O'Connell et al. 2014). Moreover, using an *ex vivo* model we observed a high expression and secretion of S100B upon demyelination, and that S100B blockade using a specific antibody reduced demyelination, astrocyte reactivity and gene expression of proinflammatory cytokines (Barateiro et al. 2016). Interestingly, active MS lesions

also showed an increase of RAGE expression by microglia/macrophages cells (Barateiro et al. 2016). These evidences suggest that S100B-RAGE interaction might contribute to MS pathogenesis.

In the present study, we examined how the use of a RAGE antagonist, that will inhibit RAGE engagement by S100B, can prevent the damage observed in our *ex vivo* demyelinating model. Our results clearly demonstrate that inhibition of S100B-RAGE interaction reduces the demyelination extent, neuronal damage and inflammatory response further supporting an important role of this axis on MS pathogenesis.

2. Materials and Methods

2.1. Animals

Animal care followed the recommendations of the European Convention for the Protection of Vertebrate Animals Used for Experimental and Other Scientific Purposes (Council Directive 86/609/EEC) and National Law 1005/92 (rules for protection of experimental animals). All animal procedures were approved by the Institutional animal care and use committee. Every effort was made to minimize the number of animals used and their suffering.

2.2. *Ex vivo* model of demyelination

To study the role of S100B-RAGE in a demyelinating event we used organotypic cerebellar slice cultures (OCSC) treated with lysophosphatidylcholine (LPC) as usual in our laboratory, which was shown to induce a great release of S100B (Barateiro et al. 2016). Cerebellum of rat pups at post-natal day 10 were isolated and cut in parasagittal slices of 400 μm using a McIlwain tissue chopper. Four slices of different animals were placed into a membrane culture insert (BD Falcon, Lincoln Park, NJ, USA) in 6-well cell culture plates in an air-liquid interface, at 37°C and 5% CO_2 conditioned atmosphere and kept in culture until 7 *days in vitro* (DIV) to allow the clearance of debris and full myelination (Birgbauer et al. 2004). After 7 DIV, slices were incubated with 0.5 mg/mL LPC in serum-free culture media, during 18h, at 37°C, in the presence or absence of a RAGE antagonist FPS-ZM1 (Calbiochem, La Jolla, CA) with a K_i = 230 nM for S100B-RAGE interaction inhibition (Deane et al. 2012). We used FPS-ZM1 in a concentration of 3 μM, more than 10x the K_i, to assure a maximal inhibition of S100B interaction with RAGE under non-toxic conditions. Following incubation, slices were maintained in fresh medium or in medium supplemented with FPS-ZM1 during 30h. Slices were collected at 9 DIV and stored in RIPA (radio-immunoprecipitation assay buffer) for protein extraction, TRIzol® reagent (Invitrogen, Carlsbad, CA, USA) at -20 °C for RNA extraction, or fixed in 4% PFA for immunohistochemistry assays.

2.3. Immunocytochemistry and data analysis

Fixed cerebellar slices were incubated with blocking solution [1 nM HEPES (Biochrom AG, Berlin, Germany), 2% heat-inactivated horse serum (Gibco, Life Technologies, Inc., Grand Island, USA), 10% heat-inactivated fetal bovine serum (Biochrom AG), 1% BSA (Sigma Chemical Co, St. Louis, MO, USA) and 0.25% Triton X-100 (Roche Diagnostics, Indianapolis, USA) in Hank's

Balanced Salt Solution (HBSS, Gibco, Life Technologies, Inc., Grand Island, USA) HBSS] for 3 h at room temperature. Slices were then incubated with primary antibodies diluted in blocking solution for 24 h, at 4°C. The following antibodies were used: neurofilament medium (NF-160, mouse, 1:200, Novocastra, Wetzlar, Germany) for neuronal axons, neural-glial antigen 2 (NG2, rabbit, 1:200, Merck Millipore, Billerica, MA, USA) for OPC, myelin basic protein (MBP, rat, 1:200, Serotec, Raleigh, NC, USA) for mature OL, glial fibrillary acidic protein (GFAP, mouse, 1:200, Novocastra) for astrocytes, calcium-binding adapter molecule 1 (Iba1, rabbit, 1:250, WAKO) for microglia, S100B (rabbit, 1:200, Abcam, Cambridge, UK) and RAGE (rabbit, 1:100, Abcam). After, slices were incubated with secondary antibodies: Alexa 594 anti-rat, Alexa 488 anti-mouse, Alexa 488 anti-rabbit (1:1000, Invitrogen), in blocking solution for 24 h, at 4 °C. To identify the total number of cells, nuclei were stained with Hoechst 33258 dye (1:1000, Sigma). Fluorescent images were acquired using a Confocal Point Scanning Microscope Zeiss LSM 710 META (Zeiss, Germany). The number of $NG2^+$ and MBP^+ cells were counted in a minimum of 200 $Hoescht^+$ nuclei from at least four independent experiments and expressed as percentage *vs.* total cell number. The percentage of the area occupied by NF-160, MBP, GFAP and Iba1 was automatically calculated using ImageJ software. Regarding myelination, the percentage of myelinated fibres was obtained by the ratio between the area of co-localization of NF-160 and MBP and the total area occupied by NF-160. Results are given by averaging values determined in the separate microscopic fields from slices of different animals.

2.4. Western Blot Analysis

Total protein extracts from slices were obtained by lysing slices in RIPA buffer, followed by sonication and centrifugation at 12,000 g for 10 min. Total protein concentrations were measured using Nanodrop ND-100 Spectrophotometer and Western blot was carried out as usual in our lab (Santos et al. 2018). The primary antibodies were: NG2 (rabbit, 1:250), MBP (rat, 1:250), S100B (rabbit, 1:500), RAGE (rabbit, 1:800), GFAP (rabbit, 1:500), pNF-κB (rabbit, 1:500, Abcam), NF-κB (rabbit, 1:500, Santa Cruz Biotechnology), or β-actin (mouse, 1:10000; Sigma). Protein bands were detected using WesternBright Sirius reagent (Advansta, Menlo Park, CA, USA) and visualized using ChemiDoc™ XRS System (Bio-Rad).

2.5. Gene expression

Total RNA was isolated from treated slices using the TRIzol® reagent method according to the manufacturer's instructions (Invitrogen) and RNA concentration was quantified using Nanodrop ND-100 Spectrophotometer (NanoDrop Technologies, Wilmington, DE, USA). Aliquots of 300 ng of total RNA were reversely transcribed using the SensiFAST™ cDNA Synthesis Kit (Bioline, MA, USA), under recommended conditions. qRT-PCR was performed on a real-time PCR detection system (Applied Biosystems 7300 Fast Real-time PCR System, Applied Biosystem, Madrid, Spain) using a SensiFAST™ SYBR No-ROX Kit (Bioline), as usual in our laboratory (Santos et al. 2018). Each sample was performed in duplicate and a non-template control was included for each gene. The sequences used as primers are listed in Table III.1.

Table III.1 - List of pairs of primers used for qRT-PCR assays.

Gene	Forward	Reverse
IL-1β	caggctccgagatgaacaac	ggtggagagctttcagctcata
MBP	agtcgcagaggacccaagat	gacaggcctctccccttttc
NG2	gggctgtgctgtctgttga	tgattcccttcaggtaaggca
PDGFRα	acgttcaagaccagcgagtt	cagtttgatggacgggagtt
PLP	cacttacagcaggtgattagagg	aaacaagagataaacaactggga
PSD-95	cgaggatgccgtggcagcc	catggctgtggggtagtcagtgcc
RAGE	tgggcaccatcttcatcattc	ggtcacccagcacaccactt
S100B	acccacatctggcagaatgag	agccatgacctttcgcattag
Synaptophysin	tcaggactcaacacctcagtgg	aacacgaaccataagttgccaa
TNF-α	tactgaacttcggggtgattggtcc	cagccttgtcccttgaagagaacc
β-actin	gctccggcatgtgcaa	aggatcttcatgaggtagt

All primers were purchased from Thermo Fisher Scientific, MA, USA.

IL-1β, interleukine-1β; MBP, myelin basic protein; NG2, neural-glial antigen 2; PDGFRα, Platelet-derived growth factor receptor α; PLP, myelin proteolipid protein; RAGE, receptor for advanced glycation endproducts; TNF-α, tumor necrosis factor-α.

2.6. Statistical Analysis

All results are presented as mean ± SEM. Differences between two groups were determined by the two-tailed t-test performed on the basis of equal and unequal variance or by one-way ANOVA for multiple comparisons, using GraphPad PRISM 6.0 (GraphPad Software, San Diego, CA, USA), as appropriate. Statistical significance was defined as a p value < 0.05.

3. Results

3.1. LPC-induced demyelination increases the expression of both S100B and RAGE that is prevented by RAGE antagonist

Since it has been described a high release of S100B in demyelinating disorders and the expression of its receptor RAGE in MS post-mortem samples (Barateiro et al. 2016), we first characterize S100B and RAGE expression upon LPC induced-demyelination of OCSC. Immunostaining of slices showed that S100B is highly expressed in response to LPC mainly by astrocytes, as indicated by the co-localization with GFAP staining, and reduced upon co-incubation with FPS-ZM1 (Figure III.1A). RAGE expression was also markedly enhanced upon demyelination, and it seems to translocate from the nucleus to the cytoplasm (Figure III.1B). However when FPS-ZM1 was added, RAGE shifted to a cell membrane position.

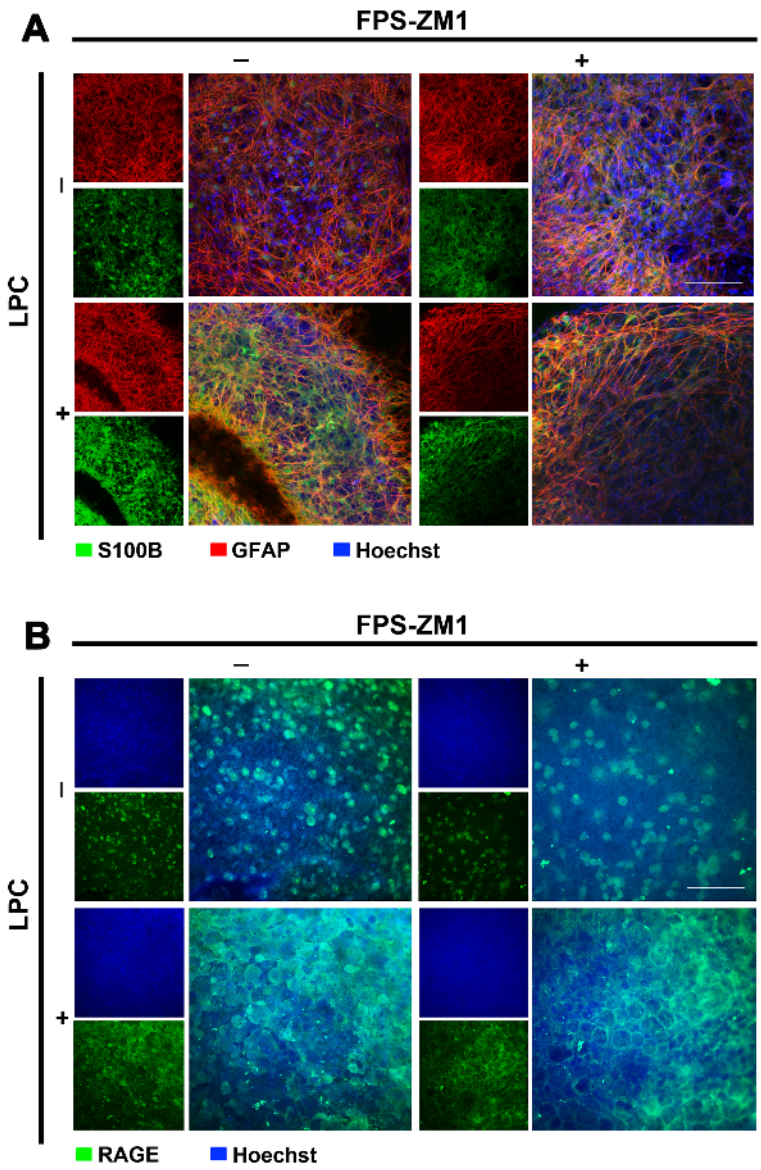

Figure III.1. LPC-induced demyelination promotes S100B and RAGE expression that is reduced by RAGE antagonist. Organotypic cerebellar slice cultures (OCSC) were exposed to lysophosphatidylcholine (LPC, 0.5 mg/mL) or LPC plus FPS-ZM1 (3 μM) at 7 *days in vitro* (DIV) for 18 h. Following 30 h of recovery OCSC were immunostained for (**A**) S100B (green) and astrocytes (GFAP) (red), or (**B**) for RAGE (green), and stained with Hoechst to detect nuclei (blue). Representative images are shown. Scale bar represents 100 μm.

In accordance, protein and gene expression of both markers showed the same pattern (Figure III.2). Both S100B and RAGE protein expression was increased even 30 h after demyelination (1.8- and 1.6-fold, $p<0.05$, respectively), whereas FPS-ZM1 co-incubation abrogated LPC effect ($p<0.05$, Figure III.2A). Also, S100B and RAGE gene expression was upregulated by LPC (1.2- and 1.5 fold, $p<0.05$, respectively) and again significantly prevented by FPS-ZM1 co-treatment ($p<0.05$, Figure III.2B). These results indicate that LPC-induced demyelination enhances the expression of both S100B and RAGE, which axis activation may contribute to associated damage.

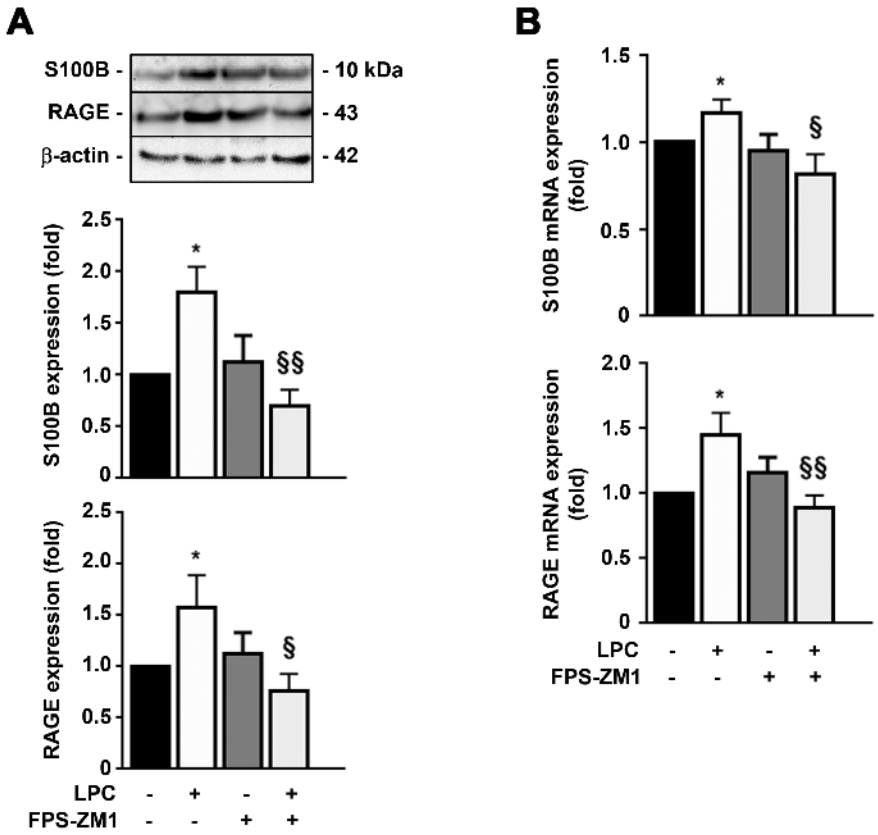

Figure III.2. LPC increases S100B and RAGE expression that is prevented by RAGE antagonist. Organotypic cerebellar slice cultures (OCSC) were exposed to lysophosphatidylcholine (LPC, 0.5 mg/mL) or LPC plus FPS-ZM1 (3 μM) at 7 *days in vitro* (DIV) for 18 h. Following 30 h of recovery, protein expression of S100B and RAGE (**A**) was evaluated by Western Blot and relative levels of the gene expression (**B**) by qRealTime-PCR. Results are mean ± SEM from at least four independent experiments. One-way ANOVA with Bonferroni multiple comparison test (*p<0.05 *vs.* control; §§p<0.01 and §p<0.05 *vs.* LPC).

3.2. LPC-induced demyelination and neuronal impairment is prevented by RAGE antagonist

We previously demonstrated in the same model of *ex vivo* demyelination that neutralization of S100B with a specific antibody partially prevents LPC-induced demyelination (Barateiro et al. 2016), highlighting the involvement of S100B in this pathogenesis. Here, we further evaluated whether S100B action was mediated by RAGE engagement. As shown in Figure III.3A-B, LPC induced a marked demyelination (0.7-fold, p<0.01) that was prevented by co-incubation with RAGE antagonist FPS-ZM1 (p<0.01), further confirming a crucial involvement of this S100B-RAGE axis in demyelination.

Neurodegeneration is other MS hallmark due either to a primary neuronal injury or as a result of myelination failure (Mandolesi et al. 2015). We observed that LPC-induced demyelination decreases the total area of NF-160 staining (0.8-fold, p<0.05) and downregulates the mRNA expression of synaptic genes PSD-95 and synapthophysin (0.4- and 0.5-fold, p<0.01, respectively, Figure III.3C-D). However, when OCSC were treated with LPC in the presence of RAGE antagonist FPS-ZM1 both neuronal integrity (1-fold, p<0.05) as well as PSD-95 and

synapthophysin gene expression (0.7- and 1.0-fold, p<0.05 and p<0.01, respectively) were recovered. Together, these results suggest that the inhibition of S100B-RAGE axis may be protective against both demyelination and axonal injury.

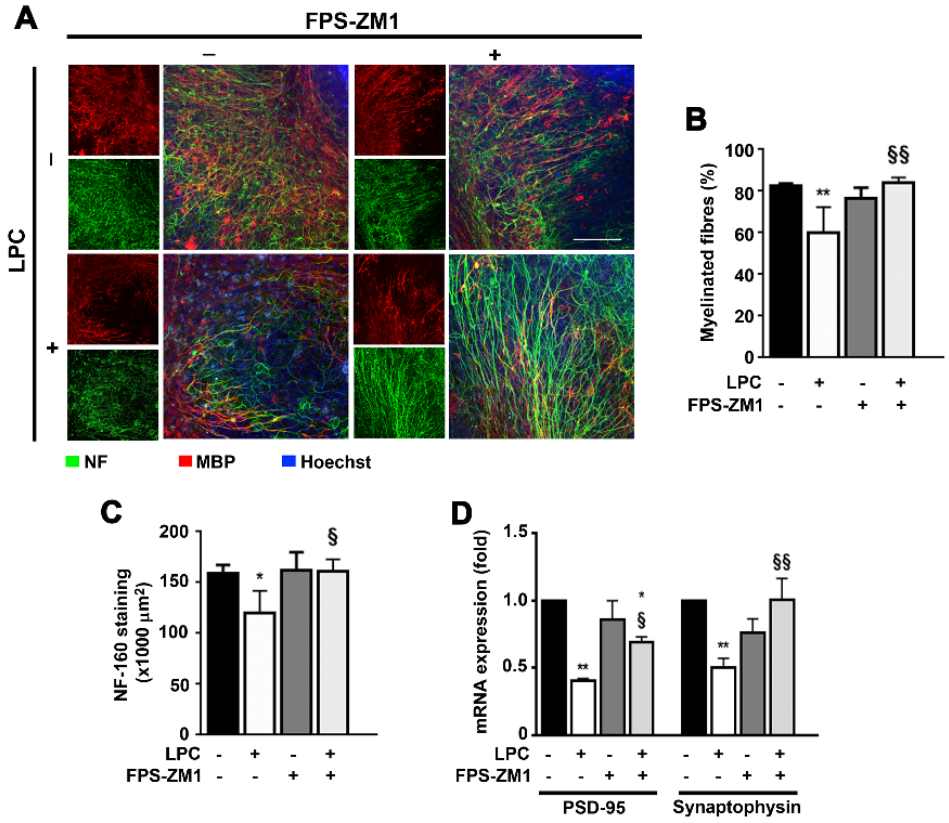

Figure III.3. LPC-induced demyelination and impairment of neuronal integrity are prevented by RAGE antagonist. Organotypic cerebellar slice cultures (OCSC) were exposed to lysophosphatidylcholine (LPC, 0.5 mg/mL) or LPC plus FPS-ZM1 (3 μM) at 7 *days in vitro* (DIV) for 18 h. Following 30 h of recovery, OCSC were immunostained for neurofilament-160 (NF-160) to detect neuronal axons (green) and myelin basic protein (MBP) to identify mature oligodendrocytes (red), and stained with Hoechst to detect nuclei (blue). (**A**) Representative images are shown. (**B**) The percentage of myelinated fibres was calculated by the ratio between the area of co-localization of NF-160 and MBP and the total area occupied by NF-160. (**C**) Axonal integrity measured by the total area of NF-160 immunostaning. (**D**) Relative levels of synaptic markers (PSD-95 and synaptophysin) were determined by qRealTime-PCR. Results are mean ± SEM from at least four independent experiments. One-way ANOVA with Bonferroni multiple comparison test (**p<0.01 and *p<0.05 *vs.* control; §§p<0.01 and §p<0.05 *vs.* LPC). Scale bar represents 100 µm.

3.3. LPC-induced impairment of oligodendrocyte differentiation is prevented by RAGE antagonist

In a previous report we showed a toxic role of high levels of S100B in OL development via RAGE activation (Santos et al. 2018), so we decided to explore in this *ex vivo* demyelination model whether S100B-RAGE axis could also be affecting OL differentiation/maturation following the demyelinating insult. As indicated in Figure III.4A-B, LPC treatment led to a significant decrease of mature OL expressing MBP (0.7-fold, p<0.01) in parallel with an increase of immature ones expressing NG2 (1.5-fold, p<0.01). Notwithstanding, co-treatment with RAGE antagonist FPS-ZM1 prevented this effect increasing the MBP^+ cells (0.9-fold, p<0.01) and partially

decreasing NG2$^+$ ones (1.3-fold, p<0.05). These results were corroborated by protein expression of these specific OL markers, as indicated in Figure III.4C. While LPC-induced demyelination increased NG2 protein expression (3-fold, p<0.05) and decreased that of MBP (0.5-fold, p<0.01), co-treatment with FPS-ZM1 prevented such effects (1.5- and 1.8-fold, p<0.05, for NG2 and MBP respectively).

Furthermore, as observed in Figure III.5, LPC-induced demyelination increased the gene expression of immature OPC markers such as PDGFRα and NG2 (1.5-fold, p<0.01), while decreased that of mature OL including MBP and PLP (0.7- and 0.6-fold, p<0.05, respectively). Co-incubation with RAGE antagonist FPS-ZM1 prevented LPC-induced effects by favouring the mRNA expression of mature markers (1.6- and 1.1-fold, p<0.01 for MBP and PLP), and inhibiting the immature ones (1.0- and 0.8-fold, p<0.05 and p<0.01 for PDGFRα and NG2).

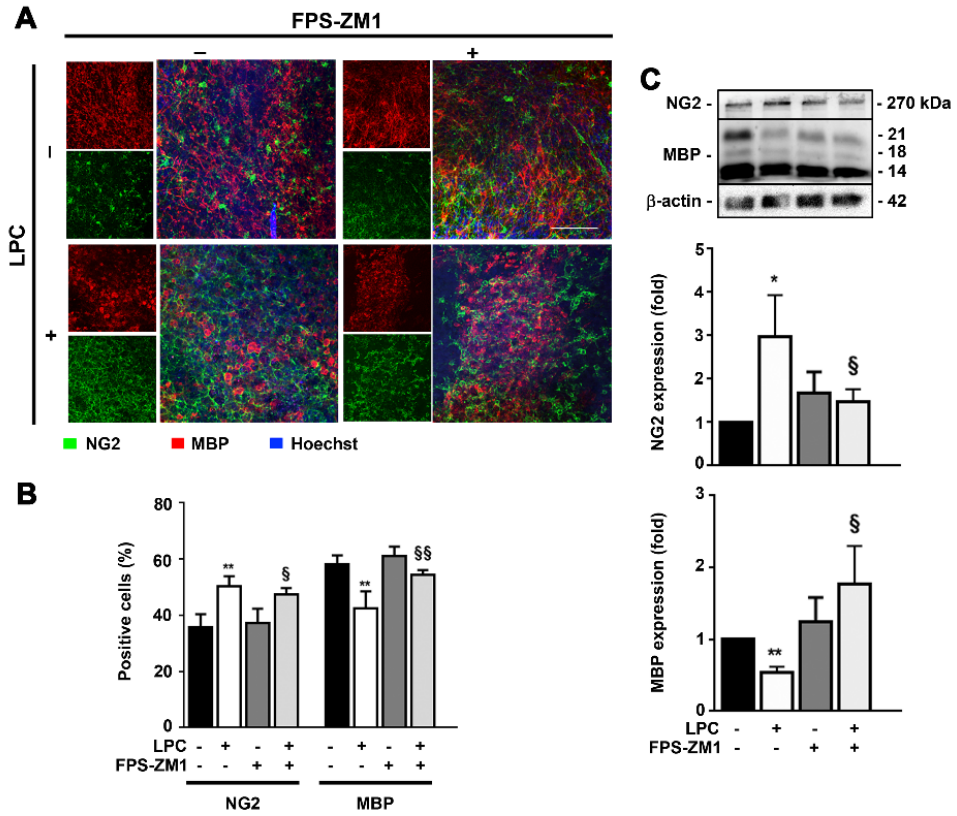

Figure III.4. LPC-induced delay in oligodendrocyte maturation is prevented by RAGE antagonist. Organotypic cerebellar slice cultures (OCSC) were exposed to lysophosphatidylcholine (LPC, 0.5 mg/mL) or LPC plus FPS-ZM1 (3 μM) at 7 *days in vitro* (DIV) for 18 h. Following 30 h of recovery, OCSC were immunostained to identify immature oligodendrocytes that express the NG2 marker (green) and mature oligodendrocytes that express myelin basic protein (MBP) (red) and stained with Hoechst to detect nuclei (blue). (**A**) Representative images are shown. (**B**) Quantification of the relative number of NG2$^+$ and MBP$^+$ cells. (**C**) Protein expression of NG2 and MBP was evaluated by Western Blot. Values are shown as mean ± SEM from at least four independent experiments performed. One-way ANOVA with Bonferroni multiple comparison test (**p<0.01 and *p<0.05 *vs.* control; §§p<0.01 and §p<0.05 *vs.* LPC). Scale bar represents 100 μm.

These data indicate that inhibition of S100B-RAGE signalling not only prevents demyelination but may also promote OL differentiation and maturation, favouring a subsequent remyelination of axonal tracts.

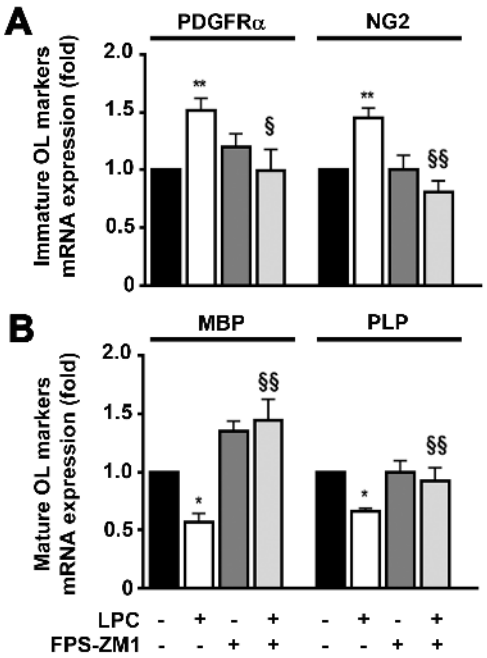

Figure III.5. LPC-induced changes in the expression of oligodendrogenesis-associated genes are prevented by RAGE antagonist. Organotypic cerebellar slice cultures (OCSC) were exposed to lysophosphatidylcholine (LPC, 0.5 mg/mL) or LPC plus FPS-ZM1 (3 μM) at 7 *days in vitro* (DIV) for 18 h. Following 30 h of recovery, relative levels of the gene expression of the (**A**) immature (PDFGRα/NG2) and (**B**) mature (MBP/PLP) oligodendrocyte markers, were determined by qRealTime-PCR. Results are mean ± SEM from at least four independent experiments. One-way ANOVA with Bonferroni multiple comparison test (**p<0.01 and *p<0.05 *vs.* control; §§p<0.01 and §p<0.05 *vs.* LPC).

3.4. LPC-induced gliosis and inflammation are prevented by RAGE antagonist

Together with demyelination and neurodegeneration, MS is characterized by a highly inflammatory response as a result of increased glia reactivity (Mallucci et al. 2015). As expected, LPC-induced demyelination promoted astrogliosis observed by an increased area of hypertrophic astrocytes with GFAP staining (1.6-fold, p<0.01, Figure III.6A-B) corroborated by enhanced protein expression (1.8-fold, p<0.05, Figure 6D), both prevented by co-exposure to RAGE antagonist FPS-ZM1 (1.1- and 1-fold, p<0.05, respectively). Interestingly, LPC also promoted a marked microgliosis, showing a more amoeboid morphology resulting in increased Iba1 staining (2.0-fold, p<0.01, Figure 6A-C) and protein expression (1.3-fold, p<0.05, Figure 6D), which was also blocked by co-treatment with RAGE antagonist (0.7-fold, p<0.05).

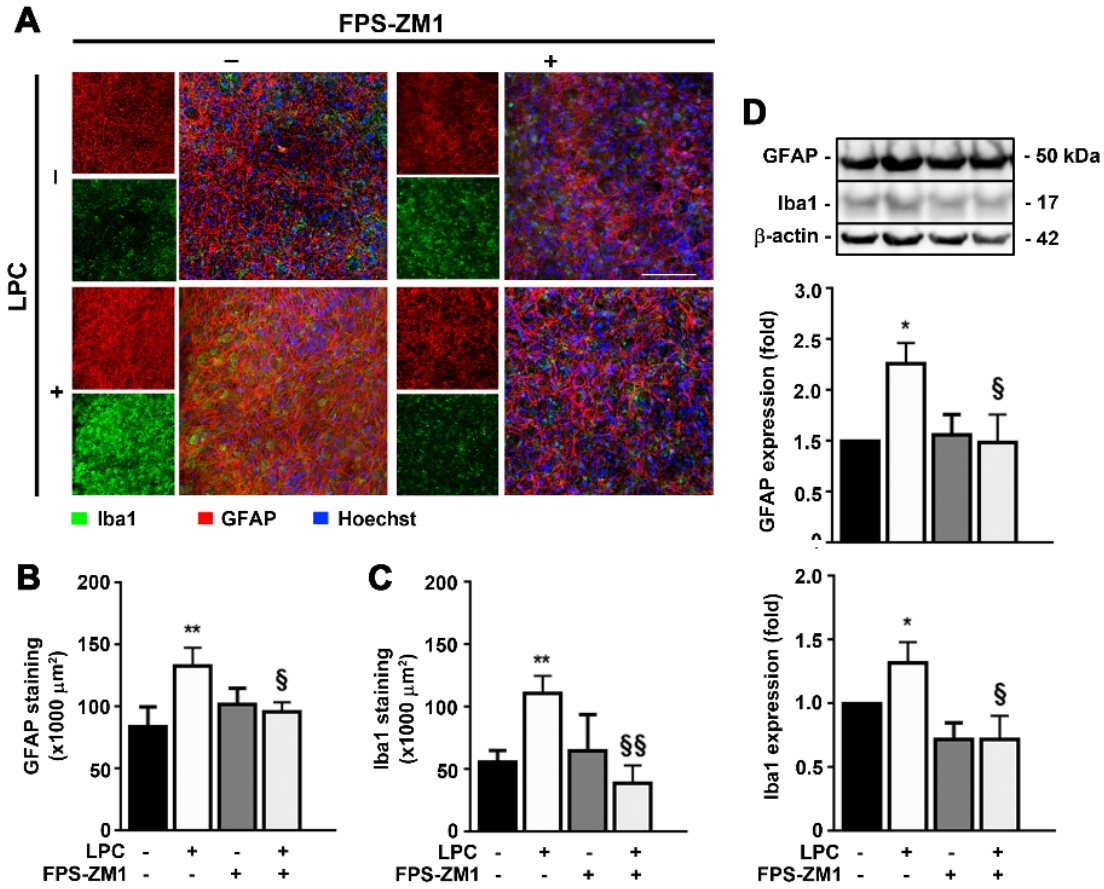

Figure III.6. LPC-induced gliosis is prevented by RAGE antagonist. Organotypic cerebellar slice cultures (OCSC) were exposed to lysophosphatidylcholine (LPC, 0.5 mg/mL) or LPC plus FPS-ZM1 (3 µM) at 7 *days in vitro* (DIV) for 18 h. Following 30 h of recovery, OCSC were immunostained for astrocytes (GFAP, red) and microglia (Iba1, green). (**A**) Representative images are shown. Gliosis were quantified by averaging the area occupied by GFAP staining (astrogliosis, **B**) and Iba1 staining (microgliosis, **C**). (**D**) Protein expression of GFAP and Iba1 was evaluated by Western Blot. Results are mean ± SEM from at least four independent experiments. One-way ANOVA with Bonferroni multiple comparison test (**p<0.01 and *p<0.05 *vs.* control; §§p<0.01 and §p<0.05 *vs.* LPC).

Given the observed gliosis we next determined the inflammatory status. NF-κB is one of the main executioners of the inflammatory response (Bianchi et al. 2010). Here we observed that LPC-induced demyelination increased the activation of NF-κB leading to a higher pNF-κB/NF-κB ratio (2.0-fold, p<0.05, Figure III.7A), while co-exposure with RAGE antagonist FPS-ZM1 prevented this NF-κB induction (0.8-fold, p<0.05). It is well established that the release of first line proinflammatory cytokines, including TNF-α and IL-1β, occurs downstream NF-κB activation. As observed in Figure III.7B-C, LPC stimulus increased both TNF-α and IL-1β mRNA expression (1.5- and 1.7-fold, p<0.05, respectively), that was also abrogated by co-treatment with FPS-ZM1 (0.8- and 0.7-fold, p<0.01, respectively).

Overall, this study indicate that blockade of S100B-RAGE axis is able to prevent gliosis and subsequent inflammatory milieu, in parallel with the rescue of myelination and neuronal/synaptic networks.

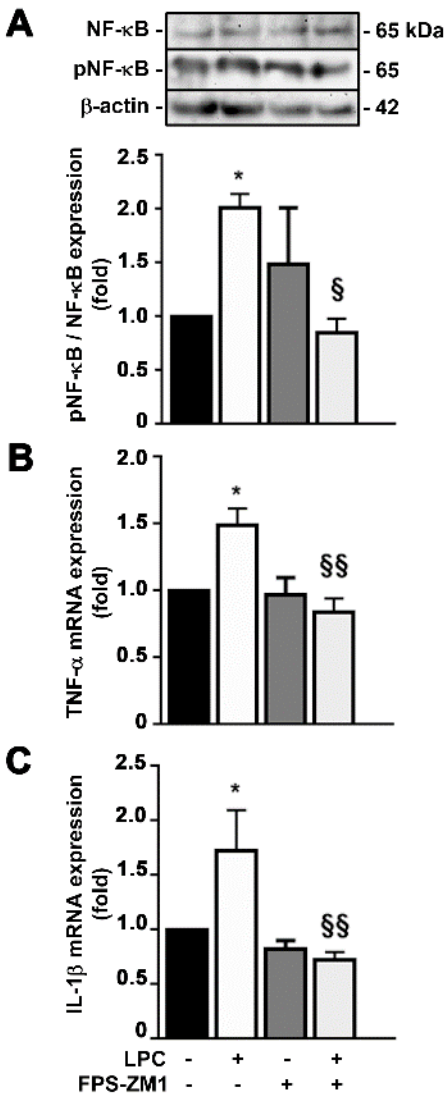

Figure III.7. LPC-induced inflammatory response is prevented by RAGE antagonist. Organotypic cerebellar slice cultures (OCSC) were exposed to lysophosphatidylcholine (LPC, 0.5 mg/mL) or LPC plus FPS-ZM1 (3 μM) at 7 *days in vitro* (DIV) for 18 h. Following 30 h of recovery, NF-κB activation was calculated by the ratio between protein expression of pNF-κB and NF-κB evaluated by Western Blot (**A**). Gene expression of TNF-α (**B**) and IL-1β (**C**) was evaluated by qRealTime-PCR. Results are mean ± SEM from at least four independent experiments. One-way ANOVA with Bonferroni multiple comparison test (*$p<0.05$ *vs.* control; $^{§§}p<0.01$ and $^{§}p<0.05$ *vs.* LPC).

4. Discussion

Excessive S100B concentration have been identified in the blood, serum and post-mortem samples of MS patients (Barateiro et al. 2016). Accordingly, S100B is markedly released particularly during acute exacerbation, reaching maximal concentration, decreasing thereafter being almost undetectable during the remission phase of the disease (Michetti et al. 1979, Massaro et al. 1985, Missler et al. 1997). Curiously, S100B levels are significantly reduced in response to mitoxantrone (Bartosik-Psujek et al. 2011) and nataluzimab (O'Connell et al. 2014). Moreover, also its receptor RAGE is found exacerbated in active lesions along the subcortical white matter or in corpus callosum (Barateiro et al. 2016), hippocampus (Sternberg et al. 2011) and spinal cord of MS patients (Yan et al. 2003). Using an *ex vivo* model of demyelination, we

also observed an increased expression of both S100B and its receptor RAGE following the demyelination stimulus of LPC. Curiously, while S100B expression was mainly due to astrocytes, RAGE expression shifted from cell nuclei to cytoplasm, suggesting an engagement of S100B-RAGE axis.

Most attractively, in the present study the use of RAGE antagonist prevented the loss of myelinated fibres induced by LPC, indicating that engagement of this receptor contributes for the extent of demyelination. These results corroborate our previous findings showing that neutralization of S100B has a beneficial role in this same model (Barateiro et al. 2016). Since S100B and RAGE are highly expressed following a demyelinating insult or in demyelinating active lesions (Barateiro et al. 2016), we may hypothesize a secondary role on the demyelination extent either by acting on OPC differentiation/maturation for proper remyelination or on the exacerbation of the inflammatory milieu and consequent neuronal damage.

S100B is expressed by OPC both in developing and adult mice brain (Deloulme et al. 2004), as well as in mature myelinating OL in brain and spinal cord (Hachem et al. 2005), being implicated in the regulation of their development. We recently showed that excessive S100B levels impair neurodevelopmental myelination (Santos et al. 2018), while its absence promoted a delayed OPC maturation following a demyelinating insult (Deloulme et al. 2004), suggesting that S100B effect may depend on the concentration locally attained. Accordingly, our results showed increased number of immature $NG2^+$ cells and decreased MBP^+ cells even after 30h upon induced demyelination, indicating a delayed or impaired OPC differentiation into mature OL, possibly impairing the needed remyelination. These results are in line with previous reports indicating that in MS patients remyelination is limited even though OPC are often efficiently recruited (Franklin 2002). Interestingly, when we blocked S100B-RAGE axis, we found a recovery of mature MBP^+ OL and a partial reduction of the immature $NG2^+$ cells, suggesting a remyelination favoring.

Along with demyelination, primary or secondary neurodegeneration, as well as synaptopathy are considered MS hallmarks (Mandolesi et al. 2015). Indeed, in post-mortem MS patient samples, the demyelinated hippocampi and cortex show decreased levels of proteins that are crucial to synaptic maintenance and function (Dutta et al. 2011). Additionally, the *in vivo* model of experimental autoimmune encephalomyelitis also exhibit structural synaptic alterations in several areas of the CNS, including the spinal cord, hippocampus, cerebellum, striatum and cortex (Mandolesi et al. 2015). In our *ex vivo* demyelinating model, we mimicked not only the impairment of axonal tracts but also the downregulation of specific pre- and post-synaptic markers gene expression. Both axonal loss and synaptic loss may be a consequence of the high S100B levels released in response to LPC treatment. In fact, deletion of S100B enhances hippocampal synaptic plasticity (Nishiyama et al. 2002), whereas excessive extracellular levels of S100B promote neuronal dysfunction or death in a direct manner (Mariggio et al. 1994), or as a result of gliosis that stimulates an inflammatory response (Hu et al. 1997, Koppal et al. 2001). Similarly, also synaptic injury or loss has been associated with astrogliosis (Zhu et al. 2003), which in our study was associated with enhanced S100B production. Together, these evidences suggest that the excessive S100B produced in response to demyelination may be implicated in the impairment of

not only oligodendrogenesis and remyelination, but also in neuronal and synaptic network dysfunction, since these effects were prevented by the use of the specific RAGE antagonist FPS-ZM1.

The marked inflammation following exacerbated gliosis is a pathological feature of MS. Our data showed that both astrogliosis and microgliosis accompanied the enhanced S100B-RAGE expression during the course of demyelination. Moreover, excessive extracellular S100B can induce an autocrine or paracrine glial activation via RAGE, leading to the transcription of proinflammatory genes (Donato et al. 2013), through the induction of NF-κB (Bianchi et al. 2010), intensifying the inflammatory response. Accordingly, we observed a marked NF-κB activation and downstream TNF-α and IL-1β gene expression upon demyelination. Once again, these effects were prevented by co-exposure to RAGE antagonist FPS-ZM1, corroborating the role of S100B-RAGE pathway in glia reactivity and in the exacerbation of the inflammatory milieu.

These results suggest that the high S100B levels released upon demyelination with consequent engagement of the S100B-RAGE axis may contribute for MS pathogenesis including impaired oligodendrogenesis and remyelination failure, neuronal loss and synaptic dysfunction, and glia activation with excessive neuroinflammatory microenvironment. Thus, blockade of S100B-RAGE interaction may be a potential new therapeutic strategy, namely for acute MS relapses where S100B is elevated. This strategy may not only prevent the inflammatory-associated damage but also accelerate OL differentiation and remyelination, as well as protecting neuronal function, leading to a reduced CNS damage and a faster recovery.

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Chapter IV

Elucidating the involvement of S100B-RAGE axis in EAE pathogenesis: role of dymethylfumarate treatment

Gisela Santos[1], Pedro Pascoal[1], Sofia Neves[3], Andreia Barateiro[1], Dora Brites[1,2], Fernanda Marques[3], João J Cerqueira[3], Adelaide Fernandes[1,2*]

[1]Neuron Glia Biology in Health and Disease, Research Institute for Medicines (iMed.ULisboa), Faculty of Pharmacy, Universidade de Lisboa, Lisboa, Portugal;

[2]Department of Biochemistry and Human Biology, Faculty of Pharmacy, Universidade de Lisboa, Lisboa, Portugal;

[3]Life and Health Sciences Research Institute, University of Minho, Braga, Portugal; ICVS/3B's - PT Government Associate Laboratory, Braga/Guimarães, Portugal

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Abstract

Multiple sclerosis (MS) is an autoimmune disease with strong inflammatory and neurodegenerative components, characterized by severe effects on motor and cognitive functions. Accordingly, most of the disease-modifying drugs used in MS therapeutics, as dimethyl fumarate (DMF), act on immune cell function in order to prevent inflammation and neurotoxicity. S100B is an inflammatory molecule mostly expressed by astrocytes upon injury and act as a signalling molecule through engagement to the receptor for advanced glycation end-products (RAGE). We recently detected high S100B levels in the cerebrospinal fluid and serum of MS patients, accompanied by increased expression of both S100B and RAGE in MS lesions of post-mortem brain samples. We also showed that S100B is highly expressed upon demyelination using an *ex vivo* model, and contributes to demyelination extent, neuronal dysfunction and inflammation. Here, we decide to evaluate the expression of S100B-RAGE axis in an *in vivo* MS model, the experimental autoimmune encephalomyelitis (EAE), and to understand if the DMF is able to alter the expression of this axis.

EAE-induced animals developed a chronic disease course from 10 days following induction, reaching a maximal clinical score of ~3-3.5 at 17 days. At this time-point animals were separated in 2 groups and treated with DMF or vehicle. Although no changes were observed in clinical score, DMF-treated animals showed moderate learning improvements. Interestingly, while EAE-induced animals showed a great loss of myelin, in parallel with decreased mRNA expression of specific oligodendrocyte and synaptic markers, DMF-treated ones showed a marked prevention of such effects. Additionally, we observed an overall increase of S100B, RAGE expression and astrogliosis, together with a higher number of $GFAP^+/S100B^+$ cells in the EAE-induced animals that decreased upon DMF treatment. The induction of S100B-RAGE axis in the EAE group was accompanied by a potentiated expression of tumor necrosis factor (TNF) -α and interleukin-1β proinflammatory cytokines and an inhibition of the interleukin-10 antiinflammatory cytokine, which pattern expression was inhibited by DMF treatment.

Overall, our results showed that EAE animals highly express S100B and RAGE, in parallel with a demyelinating, neuronal dysfunction and inflammatory phenotype. In opposite, DMF is able not only to counteract the loss of myelin fibers and synaptic markers, as expected, but also the inflammatory response, including the expression of S100B-RAGE axis. This indicate that S100B-RAGE axis is involved in the pathogenesis of the in vivo MS model, and that it may be a new and more specific target for MS therapeutic intervention.

Keywords: dimethyl fumarate (DMF), experimental autoimmune encephalomyelitis (EAE), neuroinflammation, receptor for advanced glycation endproducts (RAGE), S100B

1. Introduction

White matter provides the structural connectivity between gray matter regions throughout the brain, and the importance of myelinated systems is highlighted in both developmental and disease contexts. Myelin is critical for normal development of the structural connectivity and highly evolved cognitive functioning, enabling the rapid and efficient integrative capacity of neural systems (Filley and Fields 2016). Besides, myelination of appropriate brain regions coincides with the development of specific cognitive functions (Nagy et al. 2004, Mabbott et al. 2006). Indeed, within diseases in which myelin does not develop normally or is injured and lost, the efficiency of signal conduction is compromised resulting in a myriad of symptoms, depending on the region affected, including visual, motor and cognitive deficits (Ford et al. 2001).

Multiple sclerosis (MS) is the most common demyelinating disease of the central nervous system (CNS) characterized by loss of motor and sensory function, that results from immune-mediated inflammation, focal demyelination, extensive axonal injury (Bjartmar et al. 2001), neuronal loss (Filippi and Rocca 2005) and synaptic alterations (Centonze et al. 2009, Rossi et al. 2010). Clinically, most MS patients experience recurrent episodes (relapses) of neurological impairment with recovery in some extent, but in most cases (60-80%) the course of the disease becomes chronic and progressive with time, leading to cumulative motor disability, and cognitive deficits (Karussis 2014) that become permanent and irreversible as the disease progresses. Most interesting, defects in myelin insulation have also been associated to impaired cognitive function in 40% of MS patients (Kujala et al. 1997).

Excessive non-physiological S100B levels were described in cerebrospinal fluid, serum and post-mortem samples of MS patients (Barateiro et al. 2016). In fact, these elevated concentrations were shown to be toxic and to exacerbate the inflammatory response through glial activation with the release of proinflammatory cytokines and stress-related enzymes culminating in cell dysfunction and death (Bianchi et al. 2010, Sorci et al. 2010, Astrand et al. 2013). Several evidences suggest that these effects are dependent on the interaction of S100B with the receptor for advanced glycation endproducts (RAGE) (Ostendorp et al. 2007). Indeed, we previously showed that active MS lesions also exhibit an increased RAGE expression by microglia/macrophages cells, and that S100B is highly expressed and secreted upon demyelination, whereas its inhibition using a specific antibody reduces demyelination and an inflammatory response (Barateiro et al. 2016). Together, these evidences point to an active role of S100B-RAGE interaction in the development and progression of MS. Most attractive, S100B has also been associated with cognitive decline in hip fracture patients (Beishuizen et al. 2017) or in patients with paranoid schizophrenia (Dorofeikova et al. 2017).

In this sense, here we decided to evaluate if the expression of S100B-RAGE axis was altered in an *in vivo* model of MS, whether it could be related to the cognitive deficits and the role of dimethyl fumarate (DMF) treatment in these aspects. We choose the experimental autoimmune encephalomyelitis (EAE) since it is a widely used MS animal model characterized by inflammatory demyelination of the CNS, together with disease heterogeneity in terms of clinical course and neuropathology, as occurs in the human disease (Lucchinetti et al. 2000). In fact, depending on

the species, strain, immunization protocol and dosage of the immunogen, relapsing-remitting or chronic models can be reproduced (Gold et al. 2006, Berard et al. 2010). Moreover, EAE-induced animals have also shown memory and cognitive dysfunction (Kim et al. 2012). Our results clearly demonstrate that EAE-induced animals develop a chronic disease course that it is not ameliorated by DMT treatment at the peak of the clinical score. Nevertheless, DMF treatment improved animal cognitive performance, reduced demyelination and partially restored the deficits in gene expression of oligodendrocyte and synaptic specific markers. Interestingly, S100B-RAGE axis was highly expressed in EAE-induced animals, in parallel with a higher proinflammatory response that was also reduced in DMF-treated animals. Overall, we showed that DMF appear to have a beneficial role in EAE pathogenesis and related cognitive deficits, and that S100B-RAGE axis may also play a role in these events.

2. Materials and Methods

2.1. Animals

All experiments were conducted in accordance with the Portuguese national authority for animal experimentation, Direcção Geral de Veterinária (ID: DGV94457). Animals were housed and handled in accordance with the guidelines for the care and handling of laboratory animals in the Directive 2010/63/EU of the European Parliament and the Council. Animals were acquired from Charles River (Barcelona, Spain), housed and maintained in an SPF facility at 22-24 ºC and 55% humidity, on 12 h' light/dark cycles (lights on at 8 a.m.), and fed with regular rodent's chow and tap water *ad libitum*.

2.2. EAE induction and experimental groups

Female C57BL/6J wild-type mice were used to induce EAE. Disease induction was performed at 9-11 weeks of age, using a commercial kit (EK-2110; Hooke Laboratories, Lawrence, MA, USA), according to the manufacturer's instructions. This experiment was repeated two times. Briefly, animals were immunized subcutaneously with 200 µg of myelin oligodendrocyte glycoprotein 35-55 (MOG35-55), emulsified in complete Freund's adjuvant (CFA), at the upper and lower back (100µL of emulsion per site of injection). Pertussis toxin (PTX) in phosphate buffered saline (PBS) was administered intraperitoneal after 2 and 24 h of immunization (227 ng of PTX per injection). Non-induced age-matched female littermates were used as controls. Non-induced animals were injected subcutaneously with an emulsion of PBS in CFA (Difco Laboratories, Detroit, USA), and were injected with PTX at the same concentration and time points as the EAE animals.

Animals were daily weighted and monitored for clinical symptoms of disease, and the evaluation of the clinical disease score was performed with the experimenter blinded to the treatment group. At the clinical peak, from day 18 until day 38 post-EAE induction, animals were treated with vehicle solution, 0.8% hypromellose (Sigma Chemical Co,, Missouri, USA), or with 15 µg/g of DMF (Sigma-Aldrich) in 0.8% hypromellose by oral gavage, two times per day, accordingly to Linker and colleagues (Linker et al. 2011). Non-induced animals were treated with

vehicle solution and followed the same treatment timeline. Figure IV.1 illustrate the timeline of the experimental procedure.

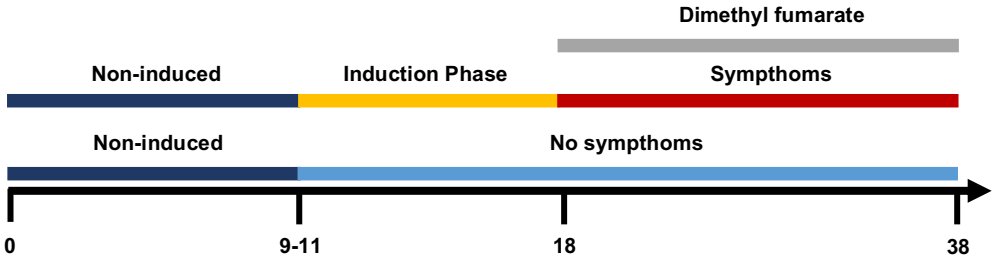

Figure IV.1. Schematic representation of the experimental procedure. Disease induction was performed at 9-11 weeks of age with myelin oligodendrocyte glycoprotein 35-55 (MOG35-55) at the day 10. Non-induced animals were injected subcutaneously with an emulsion of PBS in complete Freund's adjuvant. From day 18 until day 36 post-EAE induction, animals were treated with dimethyl fumarate and non-induced animals were treated with a vehicle solution. Both groups were sacrificed at day 38.

Disease severity was assessed as previously described (Stromnes and Goverman 2006), with few changes, as follows: 0 - no clinical symptoms; 0.5 - partially limp tail; 1 - paralyzed tail; 1.5 - at least one hind limb falls through consistently when the animal is placed on a wire rack; 2 - loss in coordinated movement, wobbly walk; 2.5 - dragging of hind limbs; 3 - paralysis of both hind limbs; 3.5 - hind limbs paralyzed and weakness of forelimbs; 4 - complete hind limbs paralysis and partial forelimbs paralysis; 4.5 - animal is not alert, no movement; 5 - moribund state or death.

2.3. Cognitive behavioural assessment – Morris water maze (MWM)

The cognitive behavioural assessment was performed during the mice-resting period (between 08h00 and 20h00). Before behavioural assessment, mice were transported to the testing room and left for habituation to room conditions for 30 minutes. Behavioural data analysis was performed with the experimenter blinded to the treatment group, and only animals with a clinical score equal or inferior to 2.5 performed the behavioural task.

To assess spatial reference memory, mice were tested in a circular pool (116 cm diameter) filled with water (24-25 ºC) placed in a dimly lit room. Titanium oxide (IV) (Sigma) was added to the water to give it a white colour, to allow the tracking system to distinguish the C57BL/6J mice from the background. The pool was divided in four imaginary quadrants, and a spatial cue was placed in the wall near each quadrant (square, stripes, triangle and a cross). A platform with 11cm of diameter was hidden in one of the quadrants (stripes). Data was collected using a fixed camera placed in the ceiling and connected to a video-tracking system (Viewpoint, Champagne-au-Mont-d'or, France).

Mice had to learn the position of the hidden platform over a period of four days. Each day, mice were placed facing the wall of the pool for a total of four trials per day. In each trial, the mice started from a different quadrant, and the sequence of quadrants was different every day. Each trial was completed whenever the mouse reached the platform or when 60 sec elapsed. Latency to reach the platform (latency to platform) and the distance swam until reaching the platform (distance to platform) was recorded for each trial during the four days. On the fifth day, the

platform was removed and a trial of 30 + 30 sec was performed (probe trial). During the probe trial, the percentage of time and of distance that each mouse swam in each quadrant was recorded to confirm the acquisition of platform location through reference memory.

2.4. Animal sacrifice

Non-induced, vehicle- and DMF-treated EAE animals were sacrificed at the light phase of the diurnal cycle, at day 38. Animals were anesthetized with an intraperitoneal injection of ketamine hydrochloride (150 mg/kg, Imalgene® 1000) plus medetomidine hydrochloride (0.3 mg/kg, Dorben®).

For histological analysis, mice were transcranially perfused with cold 0.9% saline solution, followed by perfusion with 4% paraformaldehyde. The brains were then collected, left in 4% paraformaldehyde for 24 h, and switched to a 30% sucrose solution for 24 h. Then the brains were embedded in Tissue-Tek® O.C.T.TM compound (Sakura Finetek, Japan) and snap-frozen. Using a cryostat (LEICA CM 3050S, Leica, Wetzlar, Germany) the embedded brains were cut in 20 µm coronal slides.

2.5. Histopathological analysis

Coronal slides were stained with 0.1% Luxol Fast Blue solution in 70% ethyl alcohol at 56 ºC overnight, excess stain was rinsed off with 70% ethyl alcohol and washed with distilled water for 5 min. Differentiation of the slides was done in 0.5% lithium carbonate solution for 5 min and rinsed in distilled water for another 5 min. Then slides were counterstained with hematoxylin for 10 min and washed with tap water for 5 min, 1% hydrochloric acid was then used for 5 sec to differentiate and rinsed with tap water one last time for 5 min. Finally, slides were mounted using Fluoromount-G (Southern Biotech, Birmingham, AL) for optical microscope. Images were acquired using a Leica DC 100 camera (Leica) adapted to an Axioskop microscope (Zeiss, Germany). Images were then merged using Adobe Photoshop CC 2017 software. Three specific regions usually related to demyelination were used to assess the degree of demyelination in a blinded manner for each mouse (Figure IV.2): (I) fimbria, (II) internal capsule and (III) perivascular zone. To quantify the level of demyelination we followed the standards described by Han et al. (2013): 0 - normal white matter; 1 - rare foci; 2 - a few areas of demyelination; 3 - confluent perivascular demyelination; 4 - massive demyelination involving one half of the brain; and 5 - extensive demyelination involving the whole brain.

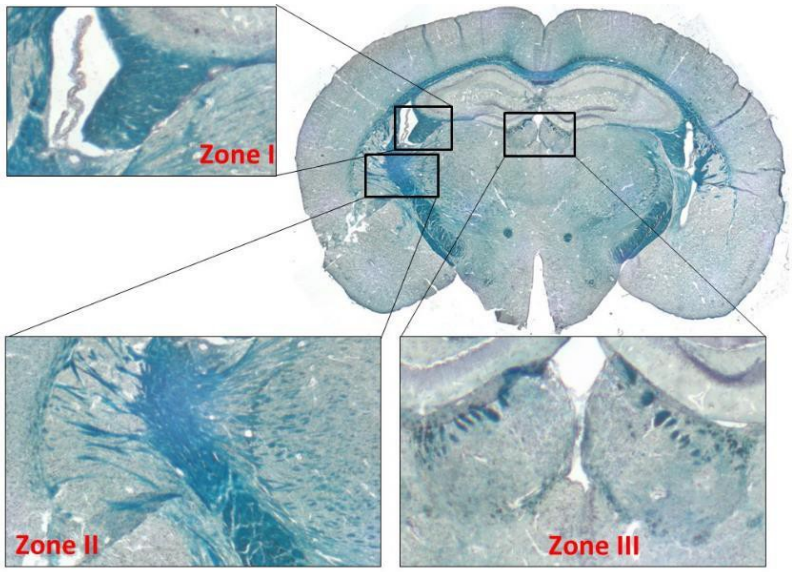

Figure IV.2. Brain coronal slices. Representative brain coronal slices (20 µm) of the experimental autoimmune encephalomyelitis induced model indicating the evaluated regions: (I) fimbria, (II) internal capsule and (III) perivascular zone.

2.6. Immunocytochemistry and data analysis

Slices were immunostained for neurofilaments to detect neuronal axons (NF-160, mouse, 1:200, Novocastra, Wetzlar, Germany), neural-glial antigen 2 (NG2) for immature oligodendrocytes (rabbit, 1:200, Merck Millipore, Billerica, MA, USA) and myelin basic proteins (MBP) for mature oligodendrocytes (rat, 1:200, Serotec, Raleigh, NC, USA), glial fibrillary acidic protein (GFAP) for astrocytes (mouse, 1:200, Novocastra), calcium-binding adapter molecule 1 (Iba1, rabbit, 1:250, WAKO) for microglia, as well as for S100B (rabbit, 1:200, Abcam, Cambridge, UK) and RAGE (rabbit, 1:100, Abcam). To identify the total number of cells, nuclei were stained with Hoechst 33258 dye (1:1000, Sigma). Fluorescent images were acquired using a Leica DMi8 Microscope (Leica) and merged using the LAS X Software for Image Acquisition (Leica). Results are given by averaging values determined in the specific regions detailed above from slices of different animals.

2.7. Gene expression

Total RNA was isolated from treated slices using the TRIzol® reagent method according to the manufacturer's instructions (Invitrogen, Carlsbad, CA, USA) and RNA concentration was quantified using Nanodrop ND-100 Spectrophotometer (NanoDrop Technologies, Wilmington, DE, USA). Aliquots of 300 ng of total RNA were reversely transcribed using the SensiFAST™ cDNA Synthesis Kit (Bioline, MA, USA), under recommended conditions. qRealTime-PCR was performed on a real-time PCR detection system (Applied Biosystems 7300 Fast Real-time PCR System, Applied Biosystem, Madrid, Spain) using a SensiFAST™ SYBR High-ROX Kit (Bioline). The qRealTime-PCR was performed in 96 well plates with each sample performed in duplicate and a non-template control was included for each gene. The sequences used as primers are listed in Supplementary Table IV.1.

qRealTime-PCR was performed under optimized conditions: 50ºC for 2 min, 95ºC for 10 min followed by 40 cycles at 95ºC for 15 s and 62ºC for 1min. In order to verify the specificity of the amplification, a melt-curve analysis was performed, immediately after the amplification protocol. Relative mRNA concentrations were calculated using the Pfaffl modification of the ΔΔCT equation, where CT is the cycle number at which fluorescence passes the threshold level of detection, taking into account the efficiencies of individual genes. The results were normalized to the housekeeping gene β-actin in the same sample and the initial amount of the template of each sample was determined as relative expression by the formula $2^{-\Delta\Delta CT}$. CT is the cycle number at which fluorescence passes the threshold level of detection, taking into account the efficiencies of individual genes; ΔCT is the value obtained for each sample performing the difference between the mean CT value of each gene of interest and the mean CT value of β-actin. ΔΔCT of one sample is the difference between its ΔCT value and the ΔCT of the sample chosen as reference.

2.8. Statistical Analysis

All results are presented as mean ± SEM. Differences between groups were determined by one-way ANOVA for multiple comparisons, using GraphPad PRISM 6.0 (GraphPad Software, San Diego, CA, USA). Statistical significance was defined as a p value < 0.05.

3. Results

3.1. Animals immunized with MOG_{35-55} developed a chronic disease course

C57Bl/6J mice were immunized with MOG_{35-55} to induce a chronic model of EAE. The EAE-induced animals presented their first clinical symptoms at day 11 reaching a maximal clinical score of 3.5 at day 17 (Figure IV.3A). At day 18, EAE-induced animals were divided in two groups, according to their clinical score and weight, in order to obtain two groups with similar average clinical score and weight, and treated with DMF or with the vehicle solution, hypromellose, until the end of the experiment. During the rest of the experiment there were no differences between the clinical score of the vehicle- and DMF-treated groups (Figure IV.3A). Non-induced animals kept a similar weight until the end of the experiment (Figure IV.3B). On the other hand, EAE-induced animals started losing weight by day 11 post-EAE induction (Figure IV.3B), when the first clinical symptoms of disease emerged (Figure IV.3A). By day 17, EAE-induced animals, independent of the treatment, had lost around 15% of their initial weight, and maintained that weight until the end of the experiment (Figure IV.3B).

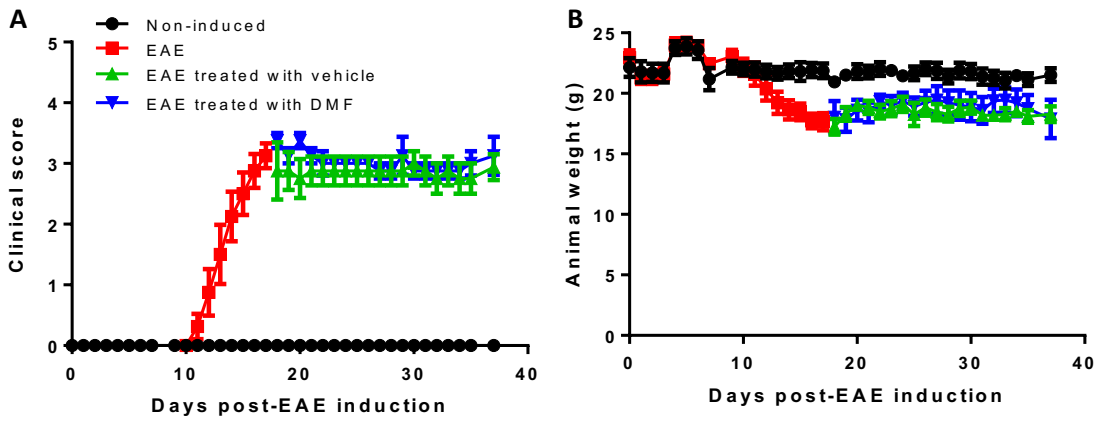

Figure IV.3. Experimental autoimmune encephalomyelitis (EAE) animals treated with vehicle or dimethyl fumarate (DMF) presented a similar disease course. (**A**) EAE animals presented their first clinical symptoms around day 11 and a chronic disease course, characterized by an increase in the clinical score until around day 17, and maintenance of similar clinical score for the remaining time of the experiment. Treatment with DMF or vehicle solution started at day 18 post-EAE induction, and both treatment groups presented a similar disease course until the end of the experiment. (**B**) Non-induced animals maintained a similar weight until the end of the experiment, whereas diseased animals started losing weight by day 11 post-EAE induction and maintained that weight until the end of the experiment.

3.2. DMF treatment improved the cognitive performance of EAE-induced animals

Non-induced and EAE animals of both treatment groups performed a reference memory task after 18 days of treatment. During the first 4 days of MWM, animals had to learn the location of a hidden platform in a swimming pool. In each day, four trials were performed, and the average of the four trials was used for statistical comparison between groups, to compare the learning curve of each group. In the fifth day, a probe trial was performed, to evaluate the animals' recollection of the platform location. During these five days, the clinical scores and weight were not different between the two EAE groups.

Throughout the four days of reference memory task, EAE animals treated with DMF presented a learning curve similar to the non-induced animals (data not shown). On the other hand, EAE animals treated with vehicle were not able to learn the location of the platform (data not shown). These results indicate that DMF treatment improved the cognitive performance in EAE-induced animals.

3.3. DMF treatment ameliorated demyelination and oligodendrocyte/synaptic markers in EAE-induced animals

Given the benefits of the DMF treatment on the learning ability of EAE-induced animals, we decided to evaluate the extension of myelination impairment in the EAE group and whether DMF treatment during the chronic phase was able to ameliorate it. To verify the demyelination degree in EAE-induced animals, brain slices were stained with Luxol Fast Blue solution and three specific regions usually related to demyelination [(I) fimbria (II) internal capsule and (III) perivascular zone] were evaluated the standards described by Han et al. (2013). As initially expected, the EAE-induced mouse group that was treated with vehicle presented a great loss of myelin comparing with non-induced animals (4.2-fold, $p<0.01$), which was prevented upon DMF treatment (~85%,

p<0.05, Figure IV.4A,B). These results were supported by gene expression analysis of immature (NG2) and mature (MBP) oligodendrocyte markers. As depicted in Figure 4C, EAE-induced animals treated with vehicle showed a marked gene downregulation of NG2 (0.04-fold, p<0.01) and MBP (0.2-fold, p<0.0001), suggesting a loss of not only mature oligodendrocyte but also of the immature oligodendrocyte pool, consistent with a chronic and long-lasting demyelination. Nonetheless, DMF treatment improved these genes expression by ~90% for NG2 (p<0.01) and ~30% for MBP (p<0.05), indicating that DMF treatment is restoring the pool of immature oligodendrocytes and improving the number of mature myelinating cells critical for remyelination.

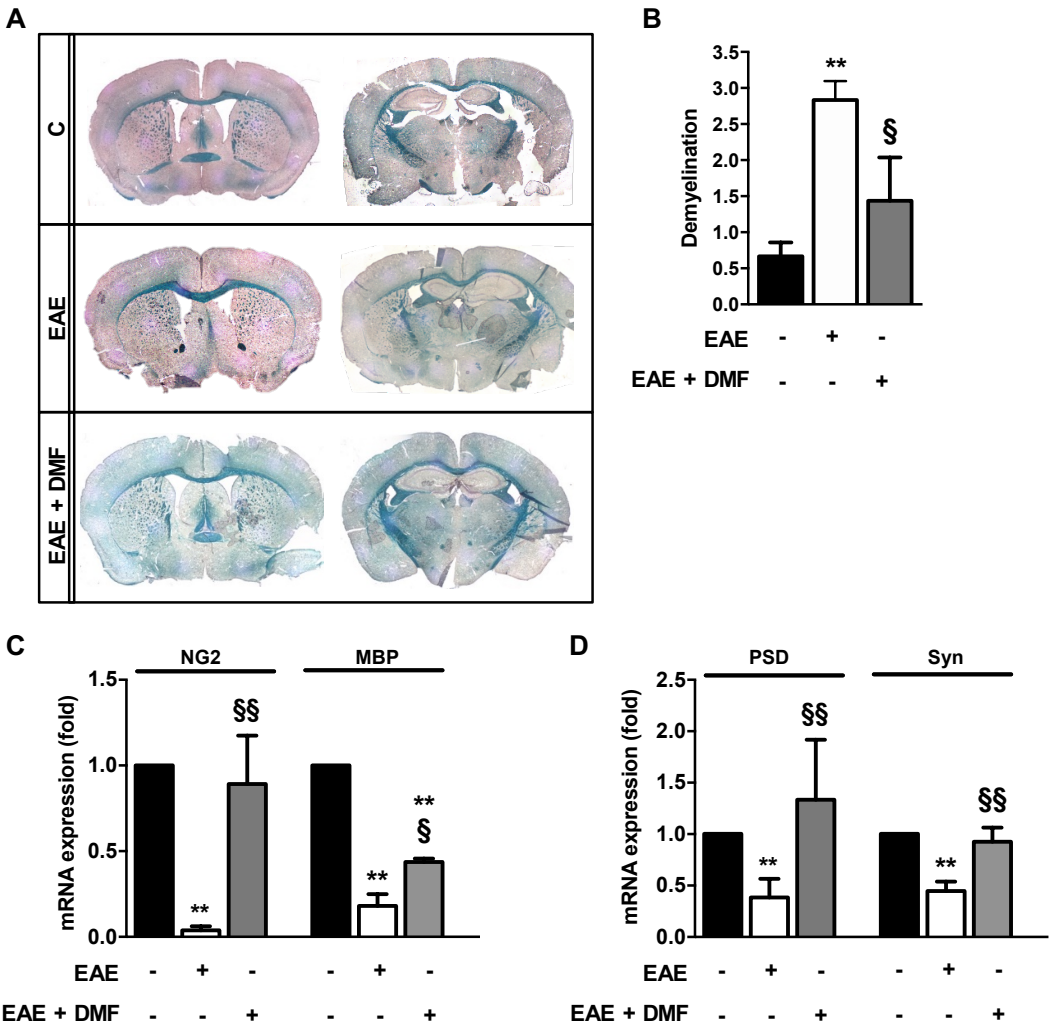

Figure IV.4. Dimethyl fumarate (DMF) treatment ameliorates demyelination and loss of specific gene expression of oligodendrocytes and synaptic markers. (**A**) Representative images of brain lesions of experimental autoimmune encephalomyelitis (EAE)-treated mouse with DMF during the chronic phase of the disease (day 38) (n=4). Luxol fast blue (myelin) staining shows a great loss of myelin in EAE group that was prevented upon DMF treatment. Bregma: 0 (Left); -1,64 (Right). (**B**) Graph bars represent a score of demyelination based on slides observation (0 - no demyelination; 5 - complete demyelination). Relative levels of immature (NG2) and mature (MBP) OL (**C**), and synaptic (**D**) markers were determined by qRealTime-PCR. Results are mean ± SEM. **p<0.01 *vs.* Control (C), and §§p<0.01 and §p<0.05 *vs.* EAE.

Interestingly, inflammation-driven synaptic abnormalities are emerging as a prominent pathogenic mechanism (Mandolesi et al. 2015) and as critical determinant of early neurodegeneration in MS, being associated with cognitive deficits (Stampanoni Bassi et al. 2017).

Thus, we next evaluated the potential protective role of DMF in synaptic integrity. EAE-induced animals treated with vehicle showed a downregulated mRNA expression of synaptic genes PSD-95 and synapthophysin (0.4- and 0.5-fold, respectively, $p<0.01$) (Figure IV.4D). However, when mice were treated with DMF both PSD-95 (1.3-fold, $p<0.05$) and synapthophysin (0.9-fold, $p<0.01$) gene expression were recovered to values similar to those observed in the non-induced group, which may justify the observed benefits related with learning capacity.

Together, these results confirm, as expected, that the EAE chronic model elicited in parallel with demyelination, an oligodendrogenesis failure accompanied by loss of synaptic markers that were partially reverted by DMF treatment.

3.4. EAE induction increased the number of cells expressing S100B, namely astrocytes, that was prevented by DMF treatment

Taking in account the massive S100B release in demyelinating disorders (Gazzolo et al. 1999, Gazzolo et al. 2001, Gazzolo et al. 2004, Huang et al. 2015, Zhou et al. 2015, Barateiro et al. 2016) and its expression along with its receptor RAGE in MS post-mortem samples (Barateiro et al. 2016), pointing to an involvement of this axis in disease pathogenesis, we next characterized the expression of S100B-RAGE axis in the chronic EAE mice. EAE-induced animals treated with vehicle showed a marked increase in the number of cells expressing S100B ranging from 1.4- to 1.9-fold in the different evaluated zones ($p<0.01$) (Figure IV.5A,B). Notwithstanding, exposure to DMF reduced the number of $S100B^+$ cells in the fimbria, internal capsule and in the periventricular zone to similar values of non-induced animals ($p<0.01$). In accordance, S100B gene expression exhibited the same pattern (Figure IV.5C). Whereas EAE-induced animals treated with vehicle showed an upregulation of S100B mRNA expression (1.5-fold, $p<0.05$) when compared to non-induced animals, the DMF-treated group presented a significantly diminished S100B expression below non-induced values ($p<0.05$).

As previously mentioned, S100B is mainly produced by glial cells, namely astrocytes (Shashoua et al. 1984, Van Eldik and Zimmer 1987). With this in mind, slices were also immunostained for GFAP, a specific astrocytic marker (Figure IV.5A,B). EAE induction promoted a shift of astrocyte morphology from a non-reactive to a reactive profile characterized by a hypertrophy of the cell body and the processes of GFAP+ cells. EAE-induced animals treated with vehicle also showed a prominent increase in the number of $GFAP^+$ astrocytic cells (1.9-fold in zone I, 2.4-fold in zone II and 2-fold in zone III, $p<0.01$) along the chronic phase. Interestingly, these animals also presented an augment number of $S100B^+/GFAP^+$ cells ($p<0.01$), more evident in the perivascular zone, suggesting that S100B is being highly produced by reactive astrocytes. Curiously, DMF treatment partially reduced the presence of a reactive astrogliosis as depict by the reduction of the $GFAP^+$ cells ($p<0.01$), together with a decrease of the $S100B^+/GFAP^+$ ones ($p<0.01$) to levels of non-induced animals, suggesting a reduction of the S100B-related inflammatory response.

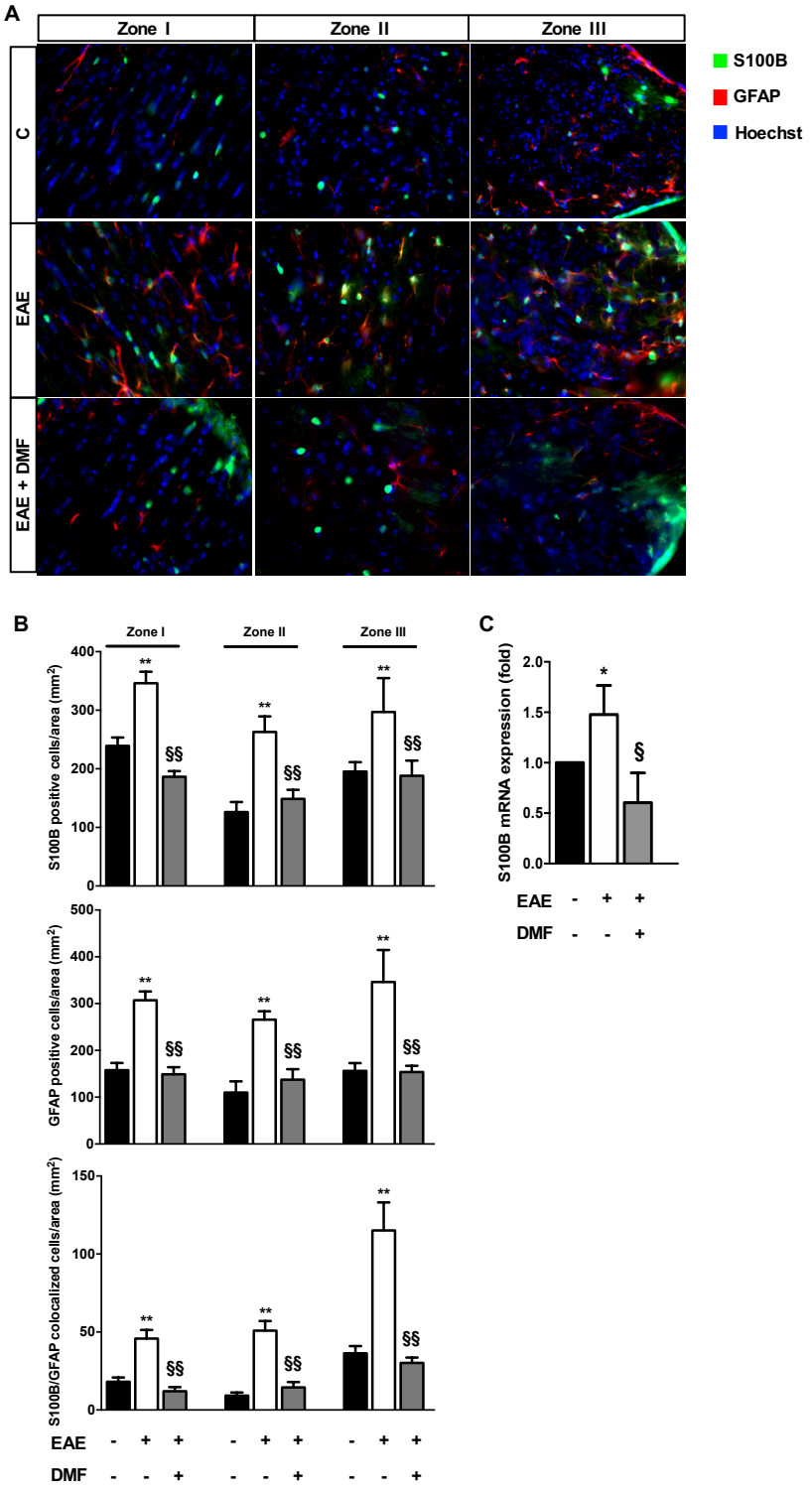

Figure IV.5. Experimental autoimmune encephalomyelitis (EAE)-induced animals have a high expression of S100B and glial fibrillary acidic protein GFAP that is prevented by dimethyl fumarate (DMF) treatment. (**A**) Representative images of brain slides of animals non-treated (C), induced for EAE or induced for EAE and treated with DMF during the chronic phase of the disease (EAE + DMF), immunostained for S100B (green) and GFAP (red) (Magnification: 40x). (**B**) Graph bars represent the density of $S100B^+$, $GFAP^+$, or $S100B^+/GFAP^+$ cells per area (mm^2). (**C**) Relative levels of S100B were determined by qRealTime-PCR. Results are mean ± SEM. **p<0.01 and *p<0.05 *vs.* respective control, and §§p<0.01 and §p<0.05 *vs.* respective EAE group.

3.5. EAE induction increased the number of cells expressing RAGE that was prevented by DMF treatment

Given our results concerning S100B expression, we decided to evaluate if its elevation was accompanied by an augment in RAGE levels. During the chronic phase of EAE, induced animals treated with vehicle showed an increased number of cells expressing RAGE in the fimbria, the internal capsule and the periventricular zone (4.8-, 2.2- and 2.5-fold, respectively, $p<0.05$), with a higher number in the last one (Figure IV.6A,B). As expected, DMF-treated animals showed a marked reduction of $RAGE^+$ cells in all studied zones: fimbria ($p<0.05$), internal capsule ($p<0.01$) and periventricular zone ($p<0.05$), to values comparable to non-induced animals. In accordance, EAE-induced animals treated with vehicle showed a slight increase in RAGE mRNA (1.3-fold), whereas DMF-treated animals presented levels of RAGE mRNA that were below non-induced animals (Figure IV.6C).

Altogether, these results showed that EAE induction leads to an enhanced expression of both S100B and RAGE, suggesting that the activation of S100B-RAGE axis may be involved in the associated pathogenesis and be modulated by DMF treatment.

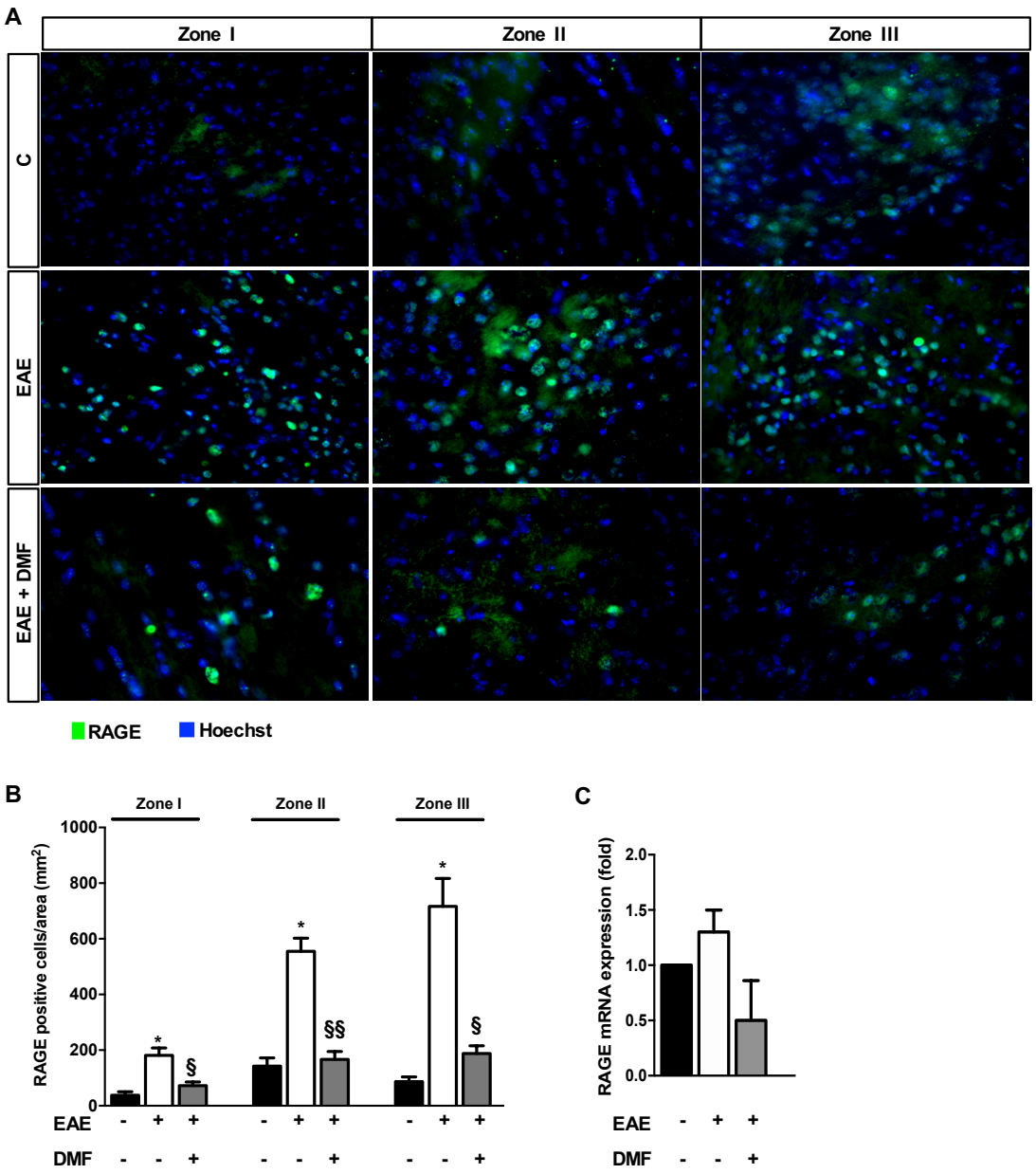

Figure IV.6. Expression of receptor for advanced glycation endproducts (RAGE) is increased in experimental autoimmune encephalomyelitis (EAE)-induced animals and is prevented by dimethyl fumarate (DMF) treatment. (**A**) Representative images of brain slides of control animals non-treated (C), induced for EAE or induced for EAE and treated with DMF during the chronic phase of the disease (EAE + DMF), immunostained for RAGE (green) and stained with Hoechst dye to detect nuclei (Magnification: 40x). (**B**) Graph bars represent the density of $RAGE^+$ cells per area (mm^2). (**C**) Relative levels of RAGE were determined by qRealTime-PCR. Results are mean ± SEM. *p<0.05 *vs.* respective C, and §§p<0.01 and §p<0.05 *vs.* EAE.

3.6. EAE induction elicited an inflammatory response that was prevented by DMF treatment

Together with demyelination and neurodegeneration, both MS and EAE are characterized by a highly inflammatory milieu in the spinal cord and the brain (Ziehn et al. 2010) as a result of the increased astrogliosis reactivity and consequent release of proinflammatory cytokines (Correale and Farez 2015). Curiously, excessive S100B concentrations may trigger microglial and astrocyte activation (Bianchi et al. 2007, Villarreal et al. 2014), which may then contribute to the release of

inflammatory and oxidative stress mediators (Bianchi et al. 2010, Sorci et al. 2010, Astrand et al. 2013). Taking in account the striking induction of S100B-RAGE axis, we decided to assess how the inflammatory response could be enhanced upon EAE induction and whether DMF treatment could modulate it.

As observed in Figure IV.7 and as expected, EAE-induced animals treated with vehicle showed a marked increase of mRNA expression of the proinflammatory cytokines tumor necrosis factor (TNF)-α and interleukin (IL)-1β (1.7- and 1.6-fold, $p<0.01$ and $p<0.05$, respectively), and decrease of the anti-inflammatory cytokine IL-10 (0.2-fold, $p<0.01$). Curiously, DMF treatment markedly reduced the inflammatory response, decreasing the expression of TNF-α and IL-1β ($p<0.01$) and increasing that of IL-10 ($p<0.01$) to similar levels of non-induced animals. Together, these data suggest that the exacerbated inflammatory response observed in the EAE-induced animals is not only a result of a potentiated release of proinflammatory cytokines, but also due to the inhibition of anti-inflammatory cytokines release. Besides, DMF is able to modulate the inflammatory environment through the reversion of the cytokines production pattern seen upon disease induction.

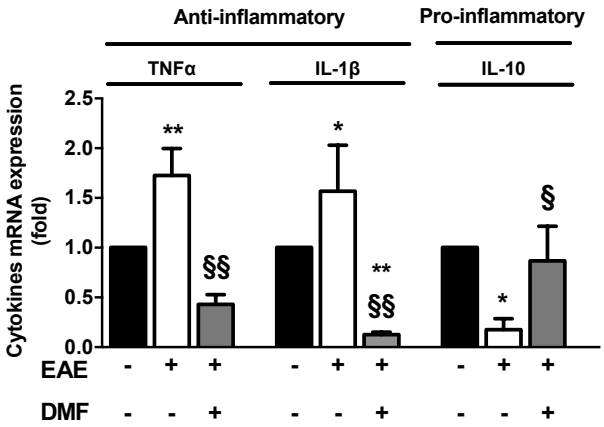

Figure IV.7. Experimental autoimmune encephalomyelitis (EAE)-induced animals have a high expression of tumor necrosis factor (TNF)-α and interleukin (IL)-1β but reduced IL-10, which are prevented by dimethyl fumarate (DMF) treatment. Animals were non-treated, induced for EAE or induced for EAE and treated with DMF during the chronic phase of the disease. Relative levels of anti- and pro-inflammatory cytokines were determined by qRealTime-PCR. Results are mean ± SEM. **$p<0.01$ and *$p<0.05$ *vs.* Control, and §§$p<0.01$ *vs.* EAE.

4. Discussion

The small molecule drug DMF (BG-12, Tecfidera®) is currently approved for the treatment of MS. Several studies suggested that DMF could improve MS pathogenesis through downregulation of T cell and myeloid cell pro-inflammatory responses (Longbrake et al. 2016, Michell-Robinson et al. 2016), while others demonstrated that DMF particularly targets pro-inflammatory mature B cell subsets (Li et al. 2017). Additional data from human and animal studies suggests both anti-inflammatory and anti-oxidant properties (di Nuzzo et al. 2014). Here we showed that DMF treatment of EAE-induced animals, although not improving the clinical score, is able to partially improve animal cognitive performance in parallel with a reduction of

demyelination, restoration of oligodendrocyte and synaptic markers and a reduction of the inflammatory response, namely of the S100B-RAGE axis.

Controversial results have been reported from the testing of DMF in the EAE model. While some authors clearly show that the clinical score observed in EAE-induced C57BL/6 female mice may be prevented by DMF treatment (100 mg/kg body weight) if given since the day of EAE induction (Schulze-Topphoff et al. 2016), others show no rescuing effect with a lower dose of DMF (30 mg/kg body weight) initiated 3 days post induction (de Bruin et al. 2016). Here, we showed no changes in the clinical scored of EAE-induced animals treated with DMF (30 mg/kg body weight) at 18 days post induction, indicating that either the lower dose or the too late treatment may have no beneficial action on the already established motor deficits. Nevertheless, DMF treatment partially improved the cognitive performance of the EAE-induced animals. Although no data have been reported on the role of DMF in the cognition of the EAE model, a recent animal study showed that the same drug regimen used in our study is able to prevent the development of early brain injury and cognitive dysfunction following subarachnoid haemorrhage if administered 2 days after the disease onset (Liu et al. 2015).

It was recently shown that patients clinically treated with DMF had increased brain magnetization transfer ratio, which correlates with the preservation or even the increase of myelin density (Arnold et al. 2014). In accordance, EAE-induced animals treated with DMF showed a decreased level of demyelination when compared to vehicle-treated EAE animals. Besides, our results also showed that upon EAE induction there is a loss of gene expression of immature NG2 and mature MBP oligodendrocyte markers, indicating a loss of premature and mature oligodendrocyte pool, both critically needed for remyelination upon injury. Interestingly, DMF treatment led to the upregulation of these oligodendrocyte markers suggesting a dual role of this compound in the protection of precursor cells and the promotion of their maturation. In accordance, DMF was previously shown to increase the differentiation of neural progenitor cells toward the oligodendrocyte lineage (Galloway et al. 2017). The mechanism behind DMF effect on oligodendrocyte maturation may rely on the regulation of their metabolism, since altered metabolism has been reported in oligodendrocytes exposed to fumarates (Huang et al. 2015, Zheng et al. 2015). Moreover, DMF attenuates overproduction of reactive oxygen species, which results in a decreased lipid peroxidation and murine oligodendrocyte protection against apoptosis and autophagy (Zarrouk et al. 2017).

Excessive S100B concentrations have been reported in the blood, serum and post-mortem samples of MS patients (Barateiro et al. 2016). The S100B protein is prominently released during an acute exacerbation, decreasing along the remission phase of the disease (Michetti et al. 1979, Massaro et al. 1985, Missler et al. 1997). Also its receptor RAGE is found excessively expressed in active lesions in subcortical white matter or corpus callosum (Barateiro et al. 2016), hippocampus (Sternberg et al. 2011) and spinal cord of MS patients (Yan et al. 2003). Accordingly, here we observed an increased expression of both S100B and its receptor RAGE during the chronic stage of the disease using the EAE model, that dramatically decrease in response to DMF treatment. Curiously, although no link has been described between DMF and

S100B-RAGE expression so far, previous studies showed that S100B levels are significantly reduced in response to mitoxantrone (Bartosik-Psujek et al. 2011) and nataluzimab (O'Connell et al. 2014), suggesting that S100B may play an important role in MS pathogenesis. In accordance, we previously showed that S100B is markedly released upon demyelination of an *ex vivo* cerebellar slice culture, and that its neutralization is able to partially prevent the associated damage and inflammatory response (Barateiro et al. 2016). On the other hand, S100B is expressed in oligodendrocyte progenitors in developing and in adult mice brain (Deloulme et al. 2004) as well as in mature myelinating oligodendrocytes in brain and spinal cord (Hachem et al. 2005), being implicated in the regulation of their development. In addition, absence of S100B led to a delayed oligodendrocyte progenitor cells maturation following cuprizone demyelinating insult in an *in vivo* model (Deloulme et al. 2004), implicating the need of physiological levels of this protein for a proper remyelination. Indeed, we recently demonstrated that while excessive S100B is able to impair oligodendrogenesis in *in vitro* and *ex vivo* models, physiological concentrations of S100B have no negative impact (Santos et al. 2018). Therefore, DMF protective role in oligodendrocytes and myelination may be partially due to the modulation of S100B-RAGE axis.

Inflammation-induced synaptic dysfunction appears in the very early phases of MS patients and in the EAE animals (Nistico et al. 2017). Interestingly, preventive or pharmacological strategies restraining proinflammatory cytokines and oxidative stress were able to rescue synaptic alterations in the EAE model (Kim et al. 2012). In this study, in parallel with the marked demyelination, we also observed a downregulation of the mRNA expression of the pre- and post-synaptic markers in EAE-induced animals. Corroborating our data, other studies showed that EAE models exhibit structural synaptic alterations in several areas of the CNS, including the spinal cord, hippocampus, cerebellum, striatum and cortex (Mandolesi et al. 2015). Curiously, DMF treatment restored the loss of synaptic marker gene expression. In accordance, a study monitoring *ex vivo* corticostriatal slices from EAE mice showed that DMF neuroprotective effect occurs through normalization of the pre-synaptic abnormalities of glutamatergic transmission (Parodi et al. 2015). Further supporting the improvements in learning abilities observed in DMF-treated animals.

Severe fibrous gliosis is a prominent pathological feature of MS and reactive astrocytes can constitute the major cell type in old completely demyelinated plaques (Norton et al. 1992), where such glial scars stain intensely for GFAP. Even though glial scars are not seen in acute EAE, they are present in chronic-relapsing models, which show persistent elevations of immunohistochemical staining for GFAP (Linington et al. 1984, Smith and Eng 1987). This phenomenon coincides with the appearance of inflammation and the onset of clinical signs (Smith et al. 1983), indicating that astrocytes become reactive when pathological changes like inflammation occur (Horstmann et al. 2013) with increases in intermediate filament expression and progressive cellular hypertrophy (Gallo and Deneen 2014, Lee and MacLean 2015). In our study, in EAE-induced mice, astrocytic cells respond to the damage by overexpressing GFAP and acquiring a hypertrophic reactive morphology, whereas DMF inhibited reactive gliosis occurrence, suggesting that DMF is able to modulate the inflammatory environment. Accordingly, some

studies showed that DMF have anti-inflammatory properties by suppressing the activation of nuclear factor (NF)-κB, which results in the inhibition of proinflammatory responses and induction of anti-inflammatory cytokines (Phillips and Fox 2013, di Nuzzo et al. 2014). Further, it has been described that the DMF active metabolite, the monomethyl fumarate, is able to shift microglia proinflammatory phenotype into a neuroprotective one through activation of the hydroxycarboxylic acid receptor 2 (Parodi et al. 2015), a receptor that is also crucial for immune cell invasion in the EAE model (Chen et al. 2014). Here, we also observed that DMF is able to prevent the EAE-associated upregulation of TNF-α and IL-1β proinflammatory cytokine mRNA expression and downregulation of that of the IL-10 anti-inflammatory molecule. So, it would be interesting to further dissect whether NF-κB and/or hydroxycarboxylic acid receptor 2 could be involved in this DMF response. To note, excessive extracellular S100B is associated to astrocytic activation and RAGE upregulation, which in turn result in activation of NF-κB (Bianchi et al. 2010) and subsequently in the transcription of proinflammatory genes (Donato et al. 2013), potentiating the inflammatory milieu. So, DMF may be regulating glia reactivity and the inflammatory response through a direct or indirect effect on S100B-RAGE axis.

Together, our results clearly show that the S100B-RAGE axis is expressed in response to EAE induction and may contribute to the MS features including cognitive dysfunction, impaired oligodendrogenesis and remyelination failure, synaptic dysfunction, astrocytic activation and inflammatory response, namely since these effects were prevented by the use of DMF which also reduced S100B-RAGE levels. Therefore, we may hypothesize that modulation of S100B-RAGE interaction may be a more specific strategy to reduce damage and improve recovery in myelin disorders related with inflammation.

5. Supplementary Material

Supplemental Table IV.1 - List of pairs of primers used for gene expression.

Gene	Forward	Reverse
IL-1β	caggctccgagatgaacaac	ggtggagagctttcagctcata
MBP	ccatccaagaagaccccaca	cccctgtcaccgctaaagaa
NG2	gggctgtgctgtctgttga	tgattcccttcaggtaaggca
PSD-95	cgaggatgccgtggcagcc	catggctgtggggtagtcagtgcc
RAGE	ttcacgacgaagttccaaacaggt	gttctaggaggactggggtg
S100B	acccacatctggcagaatgag	agccatgacctttcgcattag
Synaptophysin	tcaggactcaacacctcagtgg	aacacgaaccataagttgccaa
TNF-α	tactgaacttcggggtgattggtcc	cagccttgtcccttgaagagaacc
β-actin	gctccggcatgtgcaa	aggatcttcatgaggtagt

All primers were purchased from Thermo Fisher Scientific, MA, USA.

IL-1β, interleukine-1β; MBP, myelin basic protein; NG2, neural-glial antigen 2; RAGE, receptor for advanced glycation endproducts; TNF-α, tumor necrosis factor-α.

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Chapter V

Concluding Remarks

1. Concluding Remarks

Oligodendrocyte (OL) function and myelination are of great importance during brain development and for cognitive function. The S100B protein is expressed by OL and needed for oligodendrocyte precursor cell (OPC) differentiation and maturation (Deloulme et al. 2004). Curiously, stressed OL not only express increased levels of intracellular S100B but also its receptor for advanced glycation end-products (RAGE) (Steiner et al. 2008). Moreover, high concentrations of this protein have been described in several diseases involving OL and myelin sheath damage, as peri- and neonatal brain injuries, as well as multiple sclerosis (MS), and correlated with a poorer prognosis (Petzold et al. 2002, Bartosik-Psujek et al. 2011, Chiang et al. 2015, Huang et al. 2015, Barateiro et al. 2016, Peng et al. 2017). In this sense, the main goal of this thesis was to evaluate the role of S100B-RAGE axis in oligodendrogenesis, myelin formation and myelin injury, as well as in underlying mechanisms such as neuroinflammation, neurodegeneration and synaptopathy.

First, we decided to assess whether excessive S100B levels could affect oligodendrogenesis and if this occurred through RAGE engagement **(Chapter II)**. Excessive concentration of S100B have been described in the blood, urine and serum samples of patients with white matter damage during the peri- and neonatal period (Huang et al. 2015, Zhou et al. 2015), which are associated to a poor prognosis of premature infants with periventricular leukomalacia (Huang et al. 2015) and to a sustained brain injury of preterm infants with ischemic brain damage (Chiang et al. 2015). Therefore, it becomes crucial to understand the role of excessive S100B during neurodevelopment. We started by evaluating the effect of physiological and non-physiological concentrations of S100B in different stages of OL development, using an *in vitro* model of OPC primary cultures. The lowest S100B concentration had no effect in OL differentiation and maturation, with no alteration observed in the number of immature proteoglycan neural-glial antigen 2 $(NG2)^+$ and mature myelin basic protein $(MBP)^+$ cells, as well as in the gene expression of specific markers associated with OPC differentiation. In opposite, high extracellular S100B levels, as commonly found in several perinatal conditions (Gazzolo et al. 1999, Gazzolo et al. 2001, Gazzolo et al. 2004, Huang et al. 2015, Zhou et al. 2015), induced significant reduction of mature specific markers and MBP^+ cells, along with increased immature specific genes and $NG2^+$ cells, clearly showing a negative role of excessive S100B in oligodendrogenesis. Although basal S100B levels are needed for *in vitro* morphological transformation and maturation of preOL cells, and *in vivo* OPC maturation following a demyelinating insult (Deloulme et al. 2004), excessive concentrations might be detrimental for these cells. By using a specific RAGE antagonist, the FPS-ZM1, previously shown to prevent S100B binding to RAGE (Deane et al. 2012), we demonstrated that the toxic effect of increased extracellular S100B concentrations was dependent on RAGE engagement. Here we showed that RAGE antagonist prevented not only oligodendrogenesis impairment, by increasing the expression of mature OL specific proteins and gene markers, but also promoted mature MBP^+ cells morphological maturation, critical for the beginning of myelination. These results clearly showed that excessive non-physiological levels of

S100B have a detrimental effect on OPC differentiation and OL maturation into proper myelinating cells. In order to better understand the role of S100B-RAGE axis in the myelination process, we next used an *ex vivo* model of maturating organotypic cerebellar slice cultures (OCSC) that mimics a neonatal period in a tissue environment. This *ex vivo* model preserves the complex multicellular environment, maintaining cell relationships and the extracellular matrix in a relatively intact three-dimensional structure. Indeed, it was demonstrated that both OPC and mature OL are present in OCSC, and that compact myelin is formed (Zhang et al. 2011), indicating its suitability to evaluate the oligodendrogenesis and myelin-related processes (Ghoumari et al., 2003; Kasparov et al., 2002; Schnadelbach et al., 2001). In this thesis, we confirmed that excessive levels of S100B impaired oligodendrogenesis resulting in a reduced myelination and a compromised neuronal and synaptic integrity. In fact, axons are sustained by trophic and supporting factors released by myelinating cells (Tomassy et al., 2016), so myelination impairment during development can negatively affect the formation of neuronal networks and their synaptic integrity (Hagmann et al., 2010). Further, elevated S100B promoted an overall astrogliosis, with nuclear factor (NF)-κB induction and the emergence of an inflammation milieu. Again, the FPS-ZM1 co-treatment prevented the observed S100B-induced effects. Accordingly, previous studies showed that S100B induces astrocyte and microglia activation in a RAGE-dependent manner leading to the release of inflammatory cytokines (Bianchi et al., 2007; Villarreal et al., 2014) via the activation of NF-κB (Bianchi et al., 2010; Villarreal et al., 2014), exacerbating the inflammatory response.

Overall, these results indicate that persistently elevated S100B levels during the neurodevelopmental period have deleterious effects on myelin formation, through RAGE-dependent processes (Figure V.1), which may justify the observed brain damage and sequelae of severe inflammatory conditions of the perinatal period.

After, we decided to verify if blockade of S100B-RAGE axis, using the same RAGE antagonist, could prevent the pathogenesis observed following demyelination (**Chapter III**). With that in mind, we induced demyelination in the *ex vivo* model of mature OCSC with lysophosphatidylcholine (LPC), which is directly toxic to myelin (Birgbauer et al. 2004). After the transient demyelinating insult, OPC proliferate in response to demyelination and differentiate into mature OL that remyelinate, recapitulating a normal remyelination course. Importantly, we had previously shown that LPC-induced demyelination of the OCSC induce a great release of S100B (Barateiro et al. 2016). Here, we observed that LPC induced a marked demyelination in parallel with an increased number of immature OPC and decreased number of mature OL. Accordingly, also the protein and gene expression of specific immature and mature OL markers showed the same response to LPC treatment. Curiously, these effects were prevented in the presence of the RAGE antagonist FPS-ZM1, confirming the role of S100B-RAGE axis on the extent of demyelination and oligodendrogenesis impairment. In accordance, previous data using the same *ex vivo* model demonstrated that S100B blockade using a specific antibody reduced LPC-induced demyelination (Barateiro et al. 2016). Additionally, we also observed in LPC-treated slices a loss of neurons and

a downregulation of gene expression of synaptic markers, fundamental for neuronal and synaptic integrity. Indeed, both neurodegeneration and synaptopathy are described as MS hallmarks, which may be a consequence of the high S100B levels released in response to LPC treatment. Accordingly, the removal of S100B enhances hippocampal synaptic plasticity (Nishiyama et al. 2002), whereas excessive extracellular S100B levels promote neuronal dysfunction or death (Mariggio et al. 1994, Hu et al. 1997, Koppal et al. 2001). These evidences suggest that the excessive S100B produced in response to demyelination may be implicated in neuronal and synaptic network dysfunction, since these effects were prevented by the use of the specific RAGE antagonist FPS-ZM1. Finally, LPC also triggered glia reactivity and inflammatory response, confirmed by the increased expression of glial fibrillary acidic protein (GFAP) and ionized calcium-binding adapter molecule 1 (Iba1) proteins, morphological changes of astrocytic and microglial cells, NF-κB activation and consequent release of tumor necrosis factor (TNF)-α and interleukin (IL)-1β pro-inflammatory cytokines. Interestingly, it is known that excessive extracellular S100B induces glial activation via RAGE and the induction of NF-κB (Bianchi et al. 2010), with consequent transcription of pro-inflammatory genes (Donato et al. 2013). Again, these effects were prevented by the RAGE antagonist FPS-ZM1, corroborating the role of S100B-RAGE pathway in glial cells activation and in the exacerbation of the inflammatory environment.

Collectively, these results demonstrated that LPC-induced demyelination and associated neuronal and inflammatory damage were prevented by co-exposure to RAGE antagonist FPS-ZM1 (Figure V.1), corroborating the contribution of S100B-RAGE axis for the exacerbation of demyelination-related pathogenesis.

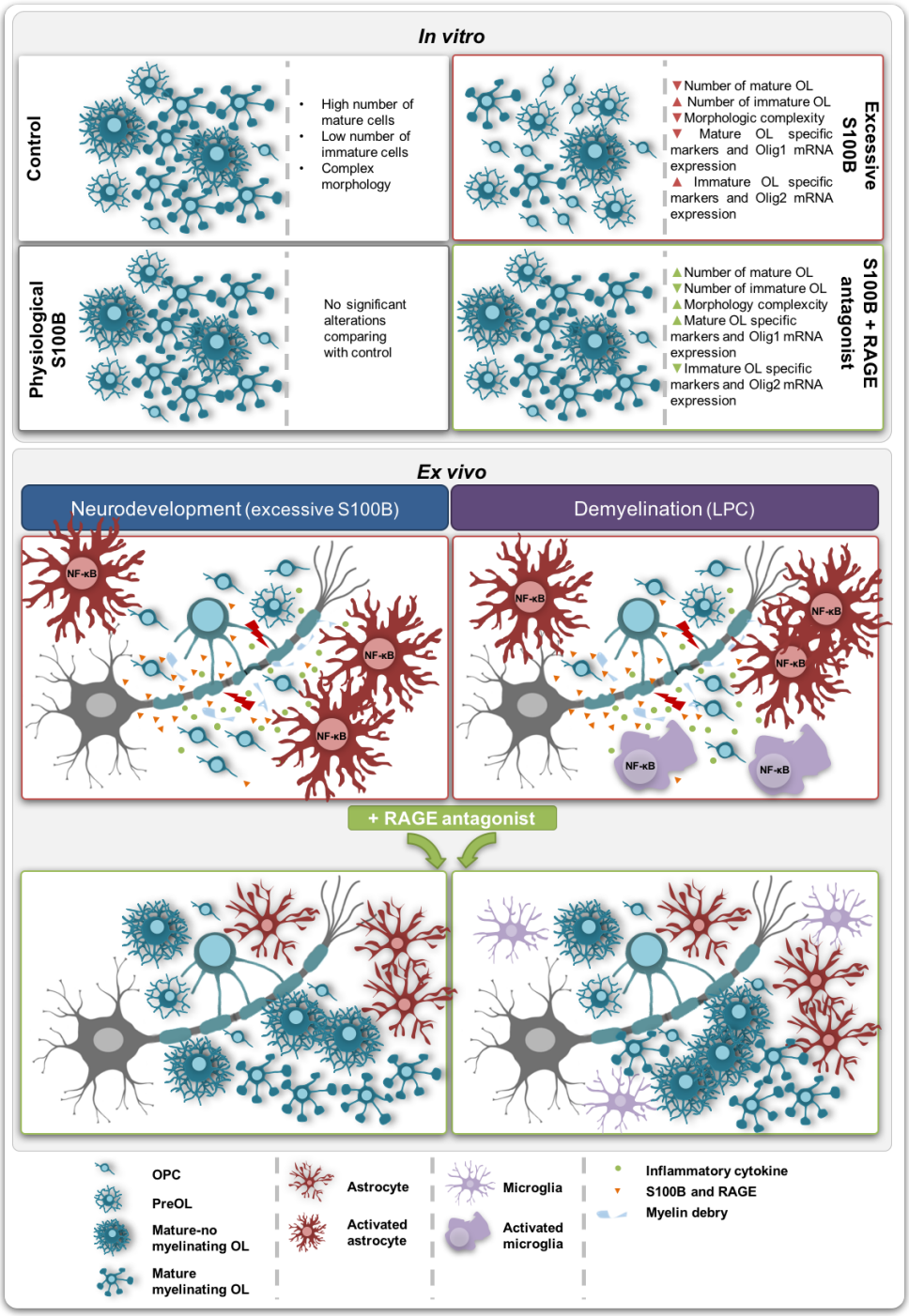

Figure V.1. Schematic representation of the major findings achieved in the Chapter II and III. Studies *in vitro* performed in oligodendrocyte precursor cell (OPC) primary cultures, showed that physiological levels of S100B do not negatively affect oligodendrogenesis, whereas high S100B concentrations induce a further S100B-RAGE axis expression that leads to decreased oligodendrocyte (OL) maturation and differentiation. Moreover, RAGE antagonist inhibits the overall negative effects of excessive S100B in OL development. In addition, *ex vivo* studies using cerebellar slice cultures, mimicking a neurodevelopmental period or a demyelination process, showed that exposure to both S100B and lysophosphatidylcholine (LPC) potentiate the S100B-RAGE axis expression, which is able to induce a decrease in OL morphological maturation and myelination, accompanied by an increased gliosis with consequent activation of nuclear factor (NF)-κB and release of pro-inflammatory cytokines, lately culminating in axonal and synaptic loss and dysfunction. Again, co-exposure with RAGE antagonist prevents the toxic effects induced by the S100B-RAGE engagement.

Taking these results into account, we then wanted to characterize the S100B-RAGE axis in the inflammatory experimental autoimmune encephalomyelitis (EAE) model and its modulation by the clinical used drug dimethyl fumarate (DMF) (**Chapter IV**). The EAE model is widely used as a MS animal model since it presents inflammatory demyelination of the central nervous system (CNS) and disease heterogeneity in terms of clinical course and neuropathology, as occurs in the human disease (Lucchinetti et al. 2000). Here we showed that EAE-induced animals develop a chronic disease course and presented cognitive performance impairment, in agreement with previous data demonstrating that this model exhibit memory and cognitive dysfunction (Kim et al. 2012). Nevertheless, DMF treatment partially improved EAE animal cognitive performance. We have also showed that EAE induction results in a high degree of demyelination and in the loss of premature and mature OL pool, whereas DMF treatment reverted these effects and induced OL maturation as previously demonstrated (Galloway et al. 2017). As described in MS (Michetti et al. 1979, Massaro et al. 1985, Missler et al. 1997, Yan et al. 2003, Sternberg et al. 2011, Barateiro et al. 2016), we observed an increased expression of both S100B and its receptor RAGE during the chronic stage of the disease using the EAE model, which dramatically decrease upon DMF treatment. Curiously, previous studies showed that S100B levels are significantly reduced in response to MS therapy (Bartosik-Psujek et al. 2011, O'Connell et al. 2014). Moreover, we also observed the loss of the synaptic markers in EAE-induced animals that were restored in response to DMF treatment, which can explain the improvement of the cognitive function. Corroborating our data, other studies showed that EAE models exhibit structural synaptic alterations (Mandolesi et al. 2015) and that DMF treatment is able to block the pre-synaptic abnormalities of glutamatergic transmission (Parodi et al. 2015). Chronic-relapsing EAE models exhibit elevations of GFAP (Linington et al. 1984, Smith and Eng 1987), which coincides with the beginning of inflammation and the onset of clinical signs (Smith et al. 1983). In our study, EAE-induced animals presented GFAP overexpression in parallel with morphological alteration, whereas DMF inhibited reactive gliosis occurrence. Here, we also observed that EAE induction results in the upregulation of TNF-α and IL-1β pro-inflammatory cytokine and downregulation of that of the IL-10 anti-inflammatory molecule, which are prevented by DMF treatment, suggesting that DMF is able to modulate the inflammatory milieu. Accordingly, DMF anti-inflammatory properties are correlated with the suppression of NF-κB activation, which results in the inhibition of pro-inflammatory responses and induction of anti-inflammatory cytokines (Phillips and Fox 2013, di Nuzzo et al. 2014).

Together, EAE-induced animals highly expressed the S100B-RAGE axis, which may be associated with the MS features since DMF prevented these effects in parallel with the reduction of S100B-RAGE levels (Figure V.2.).

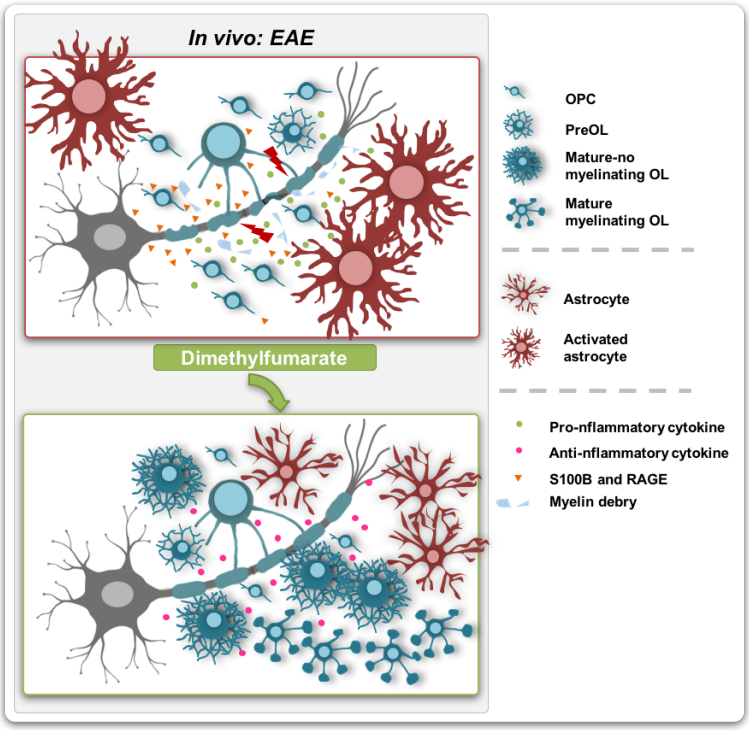

Figure V.2. Schematic representation of the major findings achieved in the Chapter IV. *In vivo* studies performed in an animal model of multiple sclerosis model, the experimental autoimmune encephalopathy (EAE), showed that S100B-RAGE axis expression is highly expressed, in parallel with decreased myelination and mature oligodendrocytes (OL), increased synaptopathy, marked gliosis and highly pro-inflammatory milieu. In opposite, the dimethyl fumarate co-treatment inhibits the overall negative effects, modulating the S100B-RAGE axis expression, increasing myelination and the pool of mature OL, promoting synaptic integrity, decreasing gliosis and the inflammatory environment. OPC, oligodendrocyte precursor cell.

Altogether, the results presented in this thesis, imply the S100B-RAGE axis in OL development and in myelin-related processes: neonatal myelination, demyelination and remyelination. Besides, this work pointed to an active role of excessive activation of the S100B-RAGE axis in several perinatal brain injury and MS features including demyelination, remyelination failure, neuronal and synaptic loss and dysfunction, glia activation and neuroinflammation. Moreover, we demonstrated that S100B-RAGE axis is highly expressed in response to OL and/or myelin injury, and that this expression is decreased after RAGE inhibition or DMF treatment, therefore suggesting that both proteins can be good biomarkers for diagnosis and prognosis, as well as to follow disease progression and to monitor therapy efficacy. Most importantly, this works supports that S100B-RAGE interaction may constitute a new and more specific target for therapeutic intervention strategies to reduce brain injury associated with inflammatory myelin-related diseases.

To note that these studies set the basis for future research that will focus on the proof of concept of the real contribution of S100B-RAGE axis for EAE pathogenesis highlighting the possible association with the psycopathological events commonly referred by the MS patients. Besides the affection of the motor and sensory systems other neuropsychiatric symptoms have already been described in MS patients reaching a prevalence of 95% (Diaz-Olavarrieta et al. 1999). A recent review summarizing the prevalence of psychiatric disorders in MS indicates that the most common are fatigue (75%), cognitive impairment (40-65%), anxiety (36%) and

depression (22.8%), suggesting a deleterious contribution of these disorders to disease pathogenesis (Haussleiter et al. 2009). Curiously, transgenic mice overexpressing human S100B have impaired learning and electrophysiological disturbances in the hippocampus (http://jaxmice.jax.org/strain/003198.html), suggesting that excessive S100B may affect cognition. So, it becomesiba of extreme importance to clarify how the presence of neuroinflammation, namely S100B, may be involved in the emergence of the psychopathological symptoms of MS and whether we may prevent them by targeting S100B, improving the quality of life of MS patients.

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